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Characterization of clonal and regenerative perivascular stem cells in human endometrium

Ruban Rex Peter Durairaj

A thesis submitted to the University of Warwick for the degree of Doctor of Philosophy.

Division of Biomedical Sciences
Warwick Medical School
University of Warwick

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been presented in any previous application for any degree.

The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

(i) Collaboration with Dr Yi-Wah Chan in the analysis of the RNA-Seq data set.

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Abstract

Decidualization denotes the transformation of endometrial stromal cells into specialised secretory decidual cells, a process indispensable for pregnancy. Decidualization of the human endometrium is not dependent on an implanting embryo but initiated during the mid-luteal phase of the cycle by elevated progesterone levels and local paracrine signals. Consequently, decidualization is a reiterative process directly linked to menstrual repair and rapid oestrogen-dependent growth. The extraordinary regenerative ability of the endometrium depends on endometrial mesenchymal stem cells (eMSCs) with inexhaustible self-renewing and differentiation capacity. Cyclic regeneration and rapid proliferation also render the stroma intrinsically heterogeneous, harbouring not only eMSCs but also endometrial transit amplifying (eTAs), mature, and senescent fibroblast subpopulations. Several lines of evidence presented in this thesis demonstrated that imbalance in these subpopulations is associated with reproductive failure. Quantification of clonal (eMSCs/eTAs) cells in mid-luteal biopsies obtained in consecutive cycles revealed increased levels in the 2nd biopsy obtained from miscarriage but not infertile patients, indicating that the tissue response to injury (i.e. the 1st biopsy) differs between patient groups. Further, in-depth characterization of primary stromal cell cultures prior to *in vitro* fertilisation (IVF) treatment showed that disordered temporal changes in the secretome of decidualizing cultures are associated with subsequent implantation failure. Additional characterization of perivascular eMSCs, which drive endometrial regeneration, highlighted the unique properties of these cells in terms of gene expression, metabolism, clonogenic and angiogenic potential. Importantly, eMSCs also formed 3D structures that resemble the uterine mucosa when cultured in Matrigel. These novel organoids termed endometrial regenerative bodies (ERBs), epithelialize when co-cultured and decidualize in response to differentiation cues. In sum, I provided evidence that dyshomeostasis between stromal subpopulations, which may be caused by eMSCs deficiency or dysfunction, precedes reproductive failure. Further, the ability of eMSCs to form ERBs provides a powerful new tool to study physiological and pathological implantation events *in vitro*.

List of Abbreviations

A.U.	Arbitrary units
ANOVA	Analysis of variance
ART	Assisted reproductive technology
BMI	Body mass index
CBP	CREB binding protein
CE	Cloning efficiency
CFU	Colony-forming unit-fibroblast
CRE	CAMP responsive element
CREB	CAMP response element binding
CREM	CAMP response element modulator
CRH	Corticotrophin releasing hormone
DIC	Differential interference contrast
EEC	Endometrial epithelial cells
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
EnSC	Endometrial stromal cells
ER	Estrogen receptor
ERB	Endometrial Regenerative Bodies
ESC	Endometrial stromal cells
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FSH	Follicular stimulating hormone
GEO	Gene Expression Omnibus
GO	Gene Ontology
GPCR	G-protein coupled receptor
H&E	Hematoxylin and eosin
HEEC	Human endometrial epithelial cells
HESC	Human endometrial stromal cells
HGF	Hepatocyte growth factor
HRE	Physoxia responsive elements

HSC	Hematopoietic stem cells
ICM	Inner cell mass
IUGR	Intrauterine growth restriction
LH	Luteinizing hormone
LIF	Leukaemia inhibitory factor
LRC	Label-retaining cells
LSGS	Low Serum Growth Supplement
MACS	Magnetic activated cell sorting
MenSC	Menstrual blood-derived stem cells
MSC	Mesenchymal stem cells
NCoR	Nuclear receptor co-repressor
OCR	Oxygen consumption rate
OPC	Oligodendrocyte progenitor cells
PAR	Protease-activated receptor
PCOS	Polycystic ovary syndrome
PLS	Partial Least Squares
PLSR	Partial least squares regression
PLZF	Promyelocytic leukaemia zinc finger
POZ	PLZF, a member of poxvirus and zinc
PR	Progesterone receptor
RCT	Randomised clinical trials
RPL	Recurrent pregnancy loss
RTCA	Real-Time Cell Analyser
SASP	Senescence-associated secretory profile
SP	Side population
TA	Transit amplifying
TF	Tissue factor

Chapter 1

1 Introduction

1.1 The human uterus

The human uterus has a single cavity formed by the fusion and canalization of basement membranes of paramesonephric (Müllerian) ducts around eight weeks of gestation. Intensive growth of uterus occurs between 16 to 24 weeks (Figure 1.1). The mesenchyme then differentiates into stroma and myometrium around 22 weeks of gestation (O'Rahilly, 1983, O'Rahilly, 1989). Uterine adenogenesis or uterine glands formation from the epithelial lining of the uterus begins in the prenatal period. A defined lumen is formed in fetal urethra, vagina, uterus, and Fallopian tubes by around 14 weeks of gestation (Terruhn, 1980). Further glandular cell proliferation is initiated after the birth, continued till puberty when glands extend from luminal epithelium to the myometrium (O'Rahilly, 1989, Shelton et al., 2012).

Placental enzymes involved in steroid production and metabolism increase the circulating levels of progesterone and oestrogen resulting in fetal exposure to steroid hormones. (Hill et al., 2010). Following birth, the levels of progesterone drop rapidly. Though the fetal endometrium is exposed to greater levels of steroid hormones than maternal tissues, it lacks decidual transformation (Brosens et al., 2015). Proliferative activity of fetal endometrium during the third trimester of pregnancy was found to be caused by placental hormones rather than ovarian hormones (Spivack, 1934). A classical study by Ober and Bernstein detailed the changes in the endometrium and ovaries from 169 fetal autopsies. The endometrium in these 169 foetuses could be classified as 68% as proliferative, 27% as secretory and 5% as progestational. More than half of the proliferative group demonstrated a resting phase whereas most of the secretory group showed an early secretory phenotype of subnuclear vacuolation. The progestational changes were similar to decidualization or menstrual breakdown (Ober and Bernstein, 1955). Increasing evidence of neonatal uterine bleeding indicates that

the stromal compartment of fetal endometrium becomes responsive to hormonal action during latter stages of pregnancy (Brosens et al., 2015).

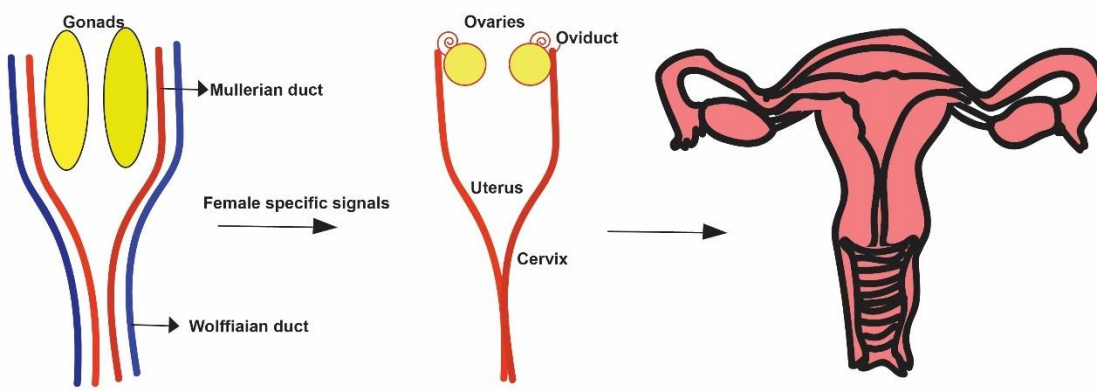

Figure 1.1. Fetal Müllerian ducts fuse to form uterus.

Urogenital ridge is bipotential, in the absence of sex-determining Y chromosome, Wolffian duct degenerates. The Müllerian ducts fuse to form a simple columnar epithelial tube that eventually differentiates into the Fallopian tubes, uterus, cervix and the upper portion of the vagina. Figure adapted from (Teixeira et al., 2008)

1.2 The human endometrium

Human endometrium, the inner lining of the uterus, is a complex and dynamic tissue as it undergoes cyclical proliferation, decidualization, shedding, restoration and regeneration for over 400 cycles throughout the reproductive lifespan of women. Every cycle is tightly regulated by ovarian steroid hormones, oestrogen and progesterone (de Ziegler et al., 1998, Jabbour et al., 2006). Following menstruation or parturition, the endometrium displays a controlled tissue remodelling, resulting in a scar-free tissue regeneration. (Salamonsen, 2003, Evans and Salamonsen, 2011).

1.3 Structure of the human endometrium

The endometrium is composed of various cell types including epithelial cells, stromal fibroblasts, leukocytes and endothelial cells. Epithelial cells within the endometrium are classified as either luminal or glandular epithelium. The endometrial stroma consists of extracellular matrix and fibroblasts, which differentiates during the decidualization process under the influence of progesterone and local paracrine signals (Gellersen & Brosens). The stroma is supported by spiral arteries and harbours resident immune populations such as macrophages and uterine natural killer cells (uNK) (Beier and Beier-Hellwig, 1998, Diedrich et al., 2007, Bulun and Adashi, 2003). The vascular compartment of the endometrium is characterised by spiral arteries surrounded by perivascular cells (Gargett et al., 2012a).

Morphologically, the endometrium can be divided into the functional and basal layers. The functionalis responds to hormonal stimuli and sloughs off after decidualization in response to falling progesterone levels in a non-conception cycle (Ferenczy and Bergeron, 1991). The functionalis also acts as the site of embryonic implantation. Ovarian hormones have a strong influence on cellular functions and morphology throughout the reproductive age of a woman resulting in cyclical regeneration and remodelling (Kabir-Salmani et al., 2008).

The basalis layer is the origin of regeneration following the menstrual destruction of the functional layer (Padykula et al., 1984). The basal layer is permanent and contains the origins of endometrial glands enclosed by dense stroma (Spencer et al., 2005). This layer is not clearly bifurcated in non-menstruating species (Gargett et al., 2012b). The histological organisation is shown in Figure 1.2.

A normal menstrual cycle lasts 28 days, though the length varies up to 35 days. The first day of bleeding is considered day 1 of the cycle. Conventionally, the menstrual cycle is divided into three phases based on uterine cycle as (i) proliferative (ii) secretory phase and (iii) menstrual phase; based on ovarian cycle as (i) Follicular phase and (ii) luteal phase.

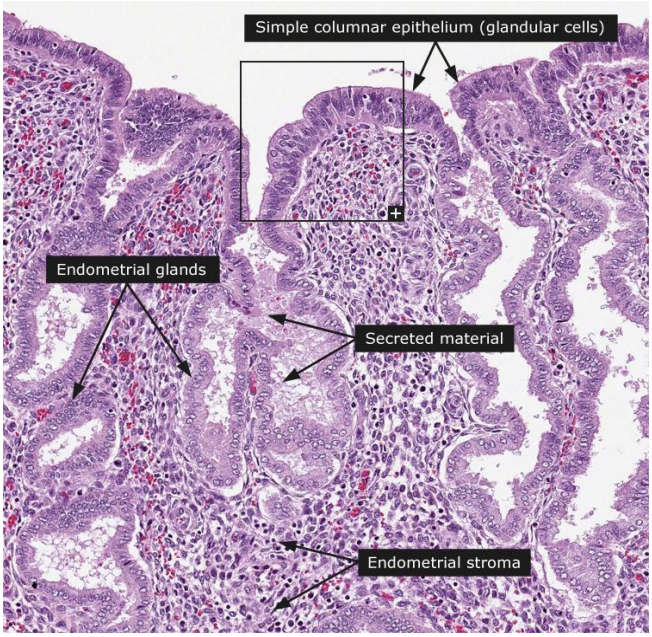

Figure 1.2. Histological organisation of endometrium

(Image is taken from www.proteinatlas.com)

1.4 Proliferative phase

The proliferative phase is marked by the regeneration of the functionalis under the influence of oestrogen, secreted by the developing follicle. During the secretory phase, endometrial stromal cells undergo a transformation, acquiring a secretory phenotype due to progesterone and oestrogen produced by corpus luteum. The menstrual phase is denoted by a sudden fall of oestrogen and progesterone due to degeneration of the corpus luteum, causing the functional layer to collapse. Endometrial responses to these ovarian hormones are achieved by specific nuclear hormone receptors present in both epithelial and stromal cells of the endometrium, along with growth factors and angiogenic factors (Sivridis and Giatromanolaki, 2004).

The menstrual cycle is a tightly controlled cellular remodelling process driven by complex interactions between the hypothalamus, pituitary, ovaries and endometrium. The hypothalamus produces gonadotropin-releasing hormone(GnRH), which regulate follicular stimulating hormone (FSH) and luteinizing hormone (LH) secretion from the pituitary. These hormones act on the ovaries and result in follicular maturation, ovulation and corpus luteum formation (Figure 1.3).

Oestrogen produced in the ovaries leads to endometrial proliferation and extensive angiogenesis. Oestrogen levels increase during two occasions during the menstrual cycle, firstly during late follicular phase before ovulation and LH surge, and secondly during the mid-luteal phase. The effect of oestrogen is exerted through oestrogen receptors alpha (ERα) and beta (ERβ), encoded by *ESR1* and *ESR2,* respectively. Oestrogen receptors are expressed by endometrial stromal cells, glandular epithelial cells, and perivascular stromal cells.

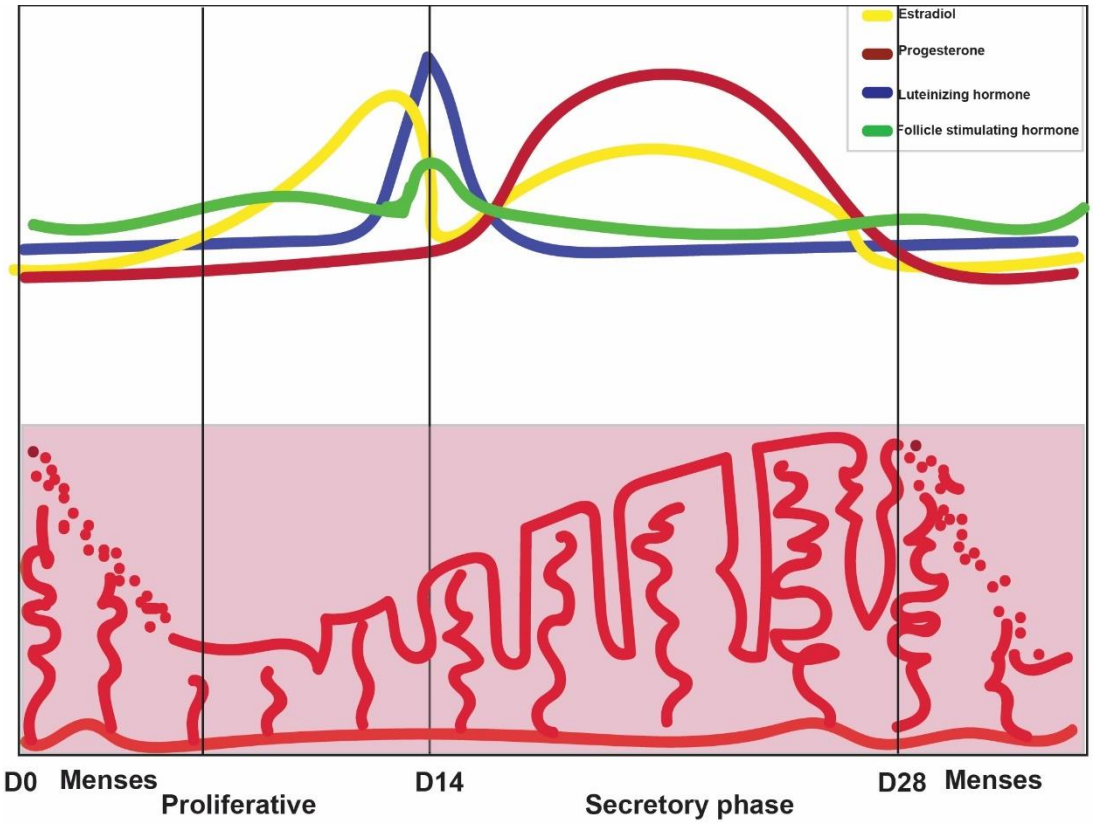

Figure 1.3. Phases of the menstrual cycle.

Sequential rise and fall of pituitary hormones regulate ovarian oestrogen and progesterone production that control the progression of the endometrium through the menstrual cycle.

1.5 Secretory Phase

Progesterone concentrations increase during the secretory phase and peak around the mid-secretory phase. Progesterone action is exerted via two of its receptor isoforms: progesterone receptor A (PR-A) and progesterone receptor B (PR-B), both encoded by single gene *PGR*. Progesterone limits the mitogenic action of oestrogen by causing differentiation of the stromal cells. Oestrogen and progesterone control PGR and its receptor isoforms. Progesterone is indispensable for the transformation of endometrial stromal cells for achieving pregnancy.

During this progesterone-induced phase of the cycle, there is an influx of immune populations including leukocytes, macrophages and neutrophils. In addition to this, vasoconstriction of spiral vessels occurs along with an increase in prostaglandin synthesis and an induction of vascular endothelial growth factor (VEGF) expression (Irwin et al., 1996). Upon progesterone withdrawal, chemokines and cytokines secreted by resident cells and immune cells cause breakdown of the ECM via secretion of various proteases including matrix metalloproteases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) (Critchley et al. 1999). The tissue damage during menstruation and the high influx of immune reactive cells are analogous to inflammation.

1.6 Decidualization

The hallmark of the menstrual cycle is the process of decidualization during which the stromal cells undergo morphological and physiological differentiation. Decidualization is induced during the mid-secretory phase of the menstrual cycle and is driven by a post-ovulatory rise in ovarian progesterone and intercellular cyclic adenosine monophosphate (cAMP) levels. Decidualization of endometrial stromal cells (ESCs) and progenitor cells is crucial for proper implantation as well as the onset of menstruation. The endometrial stromal cells differentiate into specialised secretory phenotype to provide a nutritive and immune tolerant matrix for embryo implantation

and placental formation (Brosens et al. 2002). In humans, the process of decidualization is maternally controlled and does not require any signal from the embryo. This differentiation process is pronounced in species where there is deep trophoblast invasion that breaches the maternal blood vessels (Ramsey et al. 1976, Brosens et al. 2002).

The process of decidualization starts after ovulation around day 18 with the formation of oedema in the upper stromal layer. The stromal cells surrounding the spiral arteries show cellular morphology transformation on day 23. By day 25, the oedema is replaced by large stromal cells with large nuclei (Rock and Bartlett, 1937, Gellersen and Brosens, 2014a).

Decidualization is associated with striking morphological changes. The frequent elongated fibroblastic structure changes as the nucleus become circular, the number of nucleoli increases, rough endoplasmic reticulum and Golgi bodies dilate and lipid droplets and glycogen accumulates within the cytoplasm (Kajihara et al. 2014). Prolactin (PRL) and Insulin-like growth factor binding protein-1 (IGFBP-1) are used as a marker of stromal cell differentiation cultured *in vitro*. Amniotic fluid is reported to contain massive amounts of PRL with maximum levels between 18-26 weeks of pregnancy which is produced by the decidua (Golander et al., 1978, Riddick et al., 1978). PRL production by decidualizing stromal cells starts before pregnancy (around day 21) and increases throughout the pregnancy (BRAVERMAN et al., 1984). IGFBP-1 peaks before PRL by around 16 weeks of pregnancy (Wathen et al., 1993). IGFBP-1 regulates the bioavailability of IGF-1, as this balance is crucial for normal growth and development of the foetus (Carter et al., 2004).

1.7 Role of decidualization

Decidual cells provide a better environment for trophoblast invasion compared to non-decidual cells (Gonzalez et al., 2011). Time-lapse imaging studies reveal that decidual stromal cells have a tendency to be migratory and migrate toward to the implanting

competent embryo (Weimar et al., 2012). This process of migration and attraction towards competent embryo is achieved by Elastin microfibril interfacer 1, a connective tissue glycoprotein produced by decidual stromal cells (Spessotto et al., 2006) and PDGF-AA secreted by trophoblast cells (Schwenke et al., 2013).

Endometrial stromal cells mount a tailor-made response to the single implanting embryo. This selection process either allows or rejects the implanting embryo (Teklenburg et al., 2010a, Brosens et al., 2014a). Flushing of the uterine lumen with conditioned medium of competent human embryos (i.e. resulting in a successful pregnancy following transfer) induces a highly specific gene network enriched in the major implantation and metabolic genes. By contrast, flushing with conditioned medium of poor quality human embryos triggers a stress response in the murine uterus (Brosens et al., 2014a). These observations complement other studies demonstrating that both positive and negative embryo selection takes place at implantation (Brosens et al., 2014a).

Decidualized stromal cells act as major gatekeepers and modulators of immune cells in the maternal-fetal interface. The decidua traps the uterine dendritic cells (uDCs) thus preventing them from migrating into lymph nodes for antigen presentation to cytotoxic T cells (Volchek et al., 2010). Further T cell tolerance is achieved by silencing decidual genes that encode for chemokines, by expressing lectin (galectin-1)(Barrientos et al., 2014, Bevan et al., 1994) and enzyme (indoleamine 2,3-dioxygenase) that suppress T cell action (Kudo et al., 2004).

1.8 Reprogramming during decidualization

Following decidualization, stromal cells undergo cellular reprogramming by which these cells acquire specialist functions that are needed for pregnancy. Several of the factors produced by these cells such as tissue plasminogen activator (tPA), upregulation of tissue factor (TF), the primary initiator of coagulation and plasminogen activator inhibitor 1 (PAI1) are necessary for maintaining tissue homeostasis

(Lockwood et al., 1998, Lockwood et al., 1993, Lockwood et al., 1994) and vascular integrity. An important function of decidual cells is to confer resistance to environmental stress signals. This is achieved by silencing specific stress signalling pathways such as JNK (Leitao et al., 2010, Leitao et al., 2011); upregulation of various free radical scavengers (Sugino et al., 2002a, Sugino et al., 2002b), and silencing of circadian oscillations, leading to a periodic decidual gene expression.

Marked epigenetic and transcriptional reprogramming underpins the acquisition of the decidual phenotypes as shown by some genome-wide studies (Lucas et al., 2015, Grimaldi et al., 2012, Popovici et al., 2000, Carson et al., 2002, Riesewijk et al., 2003). Even short-term exposure to deciduogenic cues can cause massive changes in gene expression (Takano et al., 2007). These changes vary in an exemplary manner as the differentiation proceeds, to be precise, the initial proinflammatory response created due to decidualization shifts to anti-inflammatory response in the course of differentiation (Salker et al., 2012a). The transcriptional changes are translated into marked protein alterations that affect most cellular compartments/functions including cell structure and motility, followed by endocytosis/exocytosis, protein biosynthesis, DNA repair, and mitosis (Paule et al., 2010).

1.9 Progesterone signalling

Ovarian progesterone is essential for female sexual characteristics, ovulation, implantation and maintenance of pregnancy throughout the gestation period (Lydon et al., 1995). PR-A is less transcriptionally active when compared to PR-B, but plays a major role functionally in the decidualization process, a notion supported by the finding that the endometrium of PR-A knockout female mice cannot mount a decidual response (Lydon et al. 1995). Progesterone binding cause a conformational change of the receptor resulting in receptor phosphorylation, dimerization and sumoylation. In fact, global cellular hypo sumoylation upon decidualization prevents PR-A sumoylation upon ligand binding and transforms the receptor from a weak to a strong

transactivator (Jones et al., 2006a). The activated receptor binds to promoter regions of progesterone-responsive genes, and recruit co-activators, co-repressors and basal transcriptional machinery (Rowan & O'Malley, 2000; Wagner et al.,1998). Over recent decades this 'classical' model of progesterone action has become more complex by the discovery of interacting transcription factors (Christian et al., 2002; Richer et al.,1998; Takano et al., 2007), targeting miRNAs (Lam et al., 2012), and rapid non-genomic progesterone signalling (Moussatche and Lyons, 2012). In addition to ovarian steroid hormone receptors, the androgen-, glucocorticoid- and mineralocorticoid receptors all have been implicated in the decidual transformation of HESCs, in vitro and in vivo (Cloke et al., 2008).

The action of progesterone is exerted via its nuclear receptors PR-A and PR-B either by activation or repression of target genes (Kastner et al., 1990). PR-A differs from PR-B by 164 N-terminal amino acids, but both isoforms display equal ligand and DNA binding affinities (Li and O'Malley, 2003). PR-A is a dominant inhibitor of PR-B and other nuclear receptors, whereas PR-B has more transcriptional activity compared to PR-A (De Mattos et al., 2004). PR-A and PR-B double knockout mice display a failed decidual response and subsequent impaired implantation (Brosens et al., 1999, Conneely et al., 2001). Isoform regulation is essential as its perturbation is linked endometrial neoplasia (Arnett-Mansfield et al., 2001). Thus, PR-A and PR-B are tightly regulated in a spatiotemporal manner throughout the menstrual cycle. PR-A is expressed by stromal cells throughout the cycle whereas its expression in epithelial cells is restricted to the proliferative phase. PR-B is expressed widely in epithelial and stromal cells during proliferative phase while decidualization downregulates its expression (Mangal et al., 1997, Mote et al., 2000, Mulac-Jericevic and Conneely, 2004).

Apart from progesterone receptors, micro RNAs (Lam et al., 2012) and promotor regulating RNAs (Lee, 2012) also play a role in governing the action of progesterone.

Progesterone binding to PR results in a conformational change which induces phosphorylation, chaperone proteins dissociation, dimerization of receptor, specific progesterone response elements (PREs) binding to target genes, and transcription machinery recruitment. Various coactivators and corepressors are involved in regulation of these steps (Wagner et al., 1998, Shibata et al., 1996).

1.10 Cyclic adenosine monophosphate (cAMP) signalling

Some stimulatory and inhibitory signals are involved in the highly timed and spatial differentiation process of decidualization. The major stimulatory signals arise from the ovarian progesterone and intercellular cAMP. Cyclic AMP is a secondary messenger molecule that is controlled through ligand binding to the G-protein coupled receptor (GPCR). Gα, a subunit of trimeric Gαβγ of the G protein, is released upon ligand binding and regulates the activity of adenylyl cyclase. Adenylyl cyclase synthesises cAMP from adenosine triphosphate (ATP). Cyclic AMP then binds to the regulatory subunits of protein kinase A (PKA), a cytoplasmic enzyme, which causes the release of catalytic subunits. These catalytic subunits bind to the nuclear targets of cAMP response element binding protein (CREB) and related cAMP response element modulator (CREM) (Skålhegg & Taskén. 2000). Phosphorylated of CREB/CREM along with co-activator CREB binding protein (CBP) binds to cAMP responsive element (CRE) leading to expression of the target genes. During decidualization, GPCR is activated by binding of luteinizing hormone/human chorionic gonadotropin (LH/hCG), corticotrophin releasing hormone (CRH), relaxin (RLX) and prostaglandin E2 (PGE2) (Tseng et al. 1992, Tang & Gurpide 1993, Frank et al. 1994, Ferrariet al. 1995).

Expression of prolactin (PRL) during late secretory phase is routinely used as a marker for decidualization as its expression is driven by the convergence of cAMP and progesterone pathways. The decidual PRL (dPRL) promoter varies from the pituitary PRL promoter (Berwaer et al. 1994, Gellersen et al. 1994) and contains a

binding site for CCAAT/enhancer-binding protein β(C/EBPβ), which is known to be crucial for cAMP-induced decidualization. C/EBPβ interacts with FOXO1a protein and activates the decidual PRL promoter. Both of these proteins were shown to accumulate in the nuclei of the decidualized stromal cells (Pohnke et al. 1999, Christian et al. 2002). *In vitro* decidualization experiments also showed that signal transducer and activator of transcription 5 (STAT5) also activates the dPRL promoter region (Mak et al. 2002).

1.11 Progesterone and cAMP signalling

Primary endometrial stromal cells express all components of progesterone signalling pathway, but only a few genes are acutely responsive to progesterone on its own. Convergence of cAMP and progesterone signalling pathways is required for complete decidualization (Figure 1.4). This convergence is accomplished via a range of mechanisms including post-translational modifications, epigenetic alterations and the action of decidualization specific transcription factors (Gellersen and Brosens, 2014a). Pre-treatment of endometrial stromal cells with cAMP followed by progesterone promotes the activity of the dPRL promoter and protein expression of PRL (Brosens et al. 1999). This suggests that cAMP sensitises the ESCs to progesterone via regulation of the PKA pathway, modifying PR function (Gellersen & Brosens, 2003). Thus, 8-bromo-cAMP, an analogue of cAMP is used along with medroxyprogesterone acetate (MPA), to differentiate stromal cells into decidualized cells, *in vitro.*

cAMP enhances the hormone-dependent transcriptional activity of PR via disruption of PR and its corepressors such as nuclear receptor co-repressor 1 and nuclear receptor co-repressor 2, and increased interaction with coactivators such as Proto-oncogene tyrosine-protein kinase Src and CREB-binding protein (Rowan et al., 2000, Wagner et al., 1998). Cyclic AMP is activated via PKA-dependent and independent pathways, also resulting in increased expression of transcription factors such as

CEBP/β, STAT5 and FOXO1, which have the ability to interact with PR (Christian et al., 2002a, Richer et al., 1998, Takano et al., 2007). FOXO1 enhances the activity of decidua specific promotor of PRL through CEBP/β via an incomplete PRE motif (Christian et al., 2002b). STAT5 induces activity in the presence of cAMP and progestin (Mak et al., 2002). The model of activated PR interacts with multiple cAMP-dependent TFs to form a multimeric complex that targets decidual genes. This was substantiated by a recent study using chromatin immunoprecipitation (ChIP) with massively parallel DNA sequencing (Lynch et al., 2011).

1.12 Cell fate decisions

Different signalling pathways are spatially and temporally coordinated in response to ovarian steroid hormones. These pathways cumulatively determine the molecular basis of endometrial stromal cellular fate, which in turn determines the quality of decidualization and receptivity. Stromal cells can shift their appropriate response based on the presence or absence of a competent or incompetent embryo. These key decisions are made by various transcription factors and signalling proteins that are present at the junctions of different signalling pathways.

For example*, PLZF,* a member of poxvirus and zinc finger (POZ)/ broad-complex, tramtrack, and bric-`a-brac (BTB) and Kruppel zinc finger (or POK) family of transcription factors, is highly conserved among mammals (Suliman et al., 2012). It is massively upregulated upon treatment with progesterone but not by cAMP. During the menstrual cycle, PLZF accumulates in the nuclei of stromal cells during mid to late secretory phase.(Fahnenstich et al., 2003). It has an antiproliferative effect and can prevent apoptosis by inhibiting proapoptotic member of B-cell lymphoma 2 (Bcl2) class of proteins (Yeyati et al., 1999, Parrado et al., 2004). This antiapoptotic response of *PLZF* is balanced by a proapoptotic cAMP-induced C-terminal fragment of Heparin-binding epidermal growth factor-like growth factor (*HB-EGF-C*). The balance between progesterone induced PLZF and HB-EGF-C is believed to be

participating in endometrial stromal fate by the balancing anti- and pro-apoptotic signals respectively (Brosens and Gellersen, 2006, Nanba et al., 2003).

FOXO1, part of multimeric transcription factor complex drives expression of the main decidual markers such as *PRL* and *IGFBP1*, following decidualization. In the absence of an implanting embryo and progesterone, FOXO1 plays a vital role in the proapoptotic pathway. Nuclear accumulation of FOXO1 is cAMP-dependent, however, upon progesterone treatment, FOXO1 translocates to the cytoplasm and is rendered inactive. Progesterone withdrawal towards the end of menstrual cycle causes rapid nuclear re-accumulation of FOXO1. This re-accumulation enables FOXO1 to target proapoptotic mediators such as Bcl-2 Interacting Mediator Of Cell Death (BIM) (Labied et al., 2006) and Fas ligand (FASLG) (Kayisli et al., 2003, Brosens and Gellersen, 2006).

Another important cell fate decision-making molecule is p53 which is stabilised by cAMP. Increased p53 levels following decidualization are hypothesised to be transcriptionally inert, however, can exert its repressive action through protein-protein interactions. Following progesterone withdrawal, transcriptional activity of p53 is activated resulting in menstrual breakdown. The balance between inert and activated p53 may serve as a key fate deciding molecule in the endometrium (Christian et al., 2002a, Brosens and Gellersen, 2006).

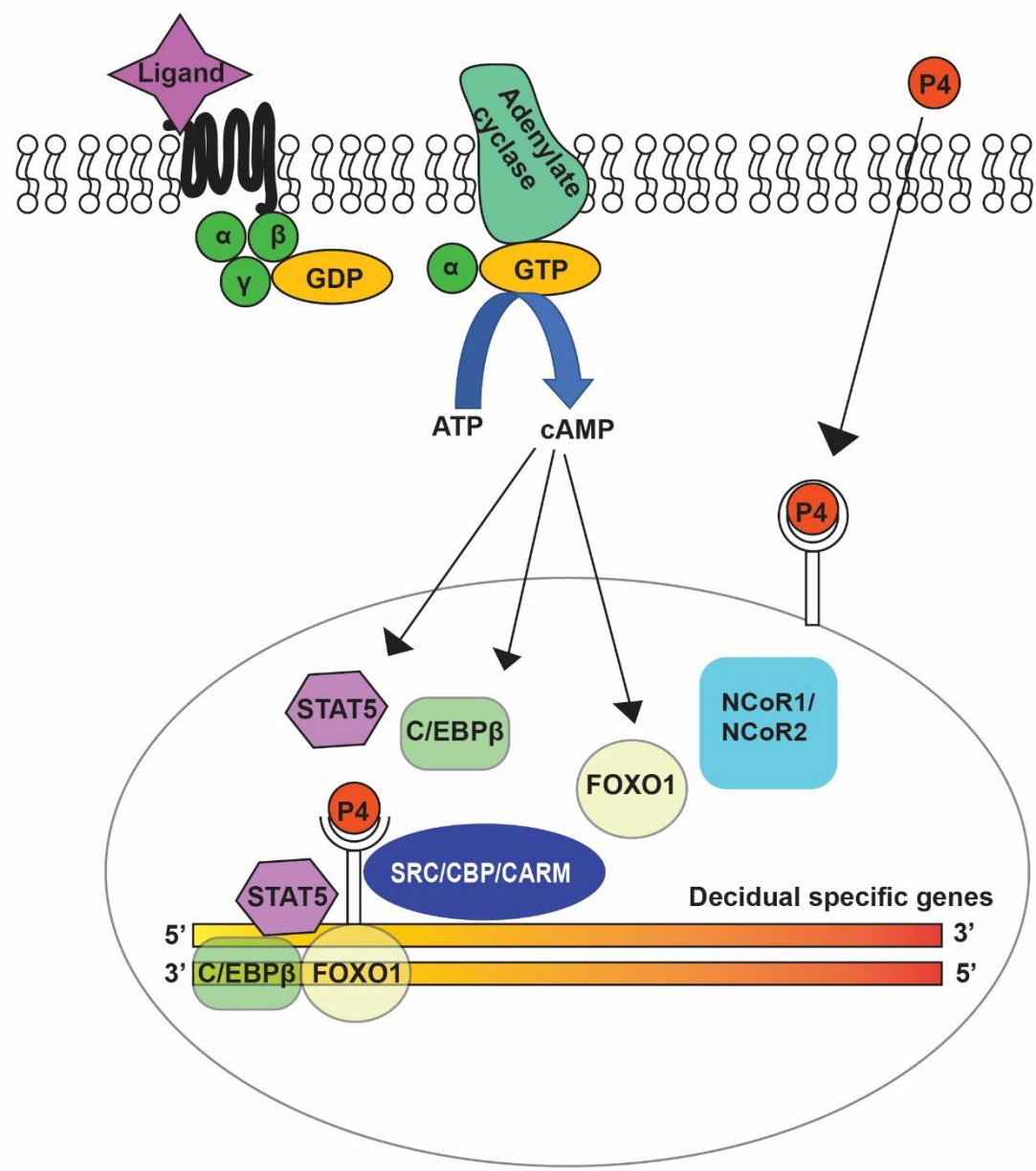

Figure 1.4. Progesterone and cAMP coordinated signalling pathway.

Ligand binding to GPCR results in an increase of secondary messenger cAMP production activating subsequent PKA-dependent and independent pathways, resulting in accumulation CEBP/β, FOXO1 and STAT5 in the nucleus. PR-A bound with progesterone interacts with these factors resulting in transcription of decidua specific genes. SRC, CBP, CARM are coactivators whereas Nuclear receptor co-repressor (NCoR)1/R2 are disrupted repressors. Figure adapted from (Gellersen and Brosens, 2003)

1.13 In vitro decidualization protocol

Various protocols have been established for decidualizing endometrial stromal cells in vitro. Among these protocols most commonly used protocols involve: (i) Estradiol and progesterone or a progesterone analogue; (ii) factors inducing cyclic AMP or an analogue and (iii) cAMP analogue in combination with progestin. The other protocols and the duration of treatments are as follows (Gellersen and Brosens, 2014b):

Table 1.1. Differentiation protocols

Treatment	Duration	Endpoint	Reference
8-bromo cyclic AMP	12-24 hours	Induction of Prolactin promotor	(Telgmann et al., 1997)
8-bromo cyclic AMP	1-3 days	mRNAs of PRL and IGFBP1	(Samalecos et al., 2009)
8-bromo cyclic AMP	2 days	Microarray analysis	(Popovici et al., 2000)
Corticotropin-releasing hormone	8 days	Prolactin secretion-enhanced with Medroxy-progesterone	(Ferrari et al., 1995)
Follicle stimulating hormone in the presence/absence of Luteinizing hormone/Human chorionic gonadotropin	4-6 days	Prolactin secretion	(Tang and Gurpide, 1993)
Medroxyprogesterone	10-20 days	PRL mRNA, IGFBP-1 mRNA	(Tseng et al., 1992)
Medroxyprogesterone and 8-bromo-cAMP	4-10 days	Prolactin secretion	(Brosens et al., 1999)
Medroxyprogesterone, estrogen and activin A	10 days	Prolactin secretion	(Jones et al., 2002)
Medroxyprogesterone, estrogen and Prostaglandin E2	3 days	Prolactin secretion	(Frank et al., 1994)
Medroxyprogesterone and Insulin-like growth factor 1	28 days	Prolactin secretion	(Rosenberg et al., 1991)
Medroxyprogesterone and Relaxin	6 days	Prolactin secretion	(Telgmann et al., 1997)

Medroxyprogesterone and Relaxin in the presence or absence of estrogen	5 days	Prolactin secretion	(RONG HUANG et al., 1987)
Medroxyprogesterone or progesterone	20 days	Prolactin secretion	(HUI ZHU et al., 1990)
Progesterone + cortisone+ 8-bromo-cAMP	4 days	PRL mRNA, IGFBP-1 mRNA	(Kuroda et al., 2012)
Progesterone + DES + epidermal growth factor	25 days	Prolactin secretion	(IRWIN et al., 1991)
Progesterone + DHT+ 8-bromo-cAMP	4-8 days	Prolactin and IGFBP-1 secretion and prolactin mRNA	(Cloke et al., 2008)
Progesterone + Estrogen	10-15 days	Prolactin secretion	(Irwin et al., 1989)
Progesterone + Estrogen	14 days	IGFBP-1 secretion	(Hess et al., 2007)
Progesterone + Estrogen + epidermal growth factor	10 days	Microarray analysis	(Popovici et al., 2000)
Progesterone + Estrogen + Interleukin-11	12 days	Prolactin and IGFBP-1 secretion	(Dimitriadis et al., 2002)

The duration and experimental design varies between these *in vitro* studies however these treatments cause the cellular reprogramming required to acquire specialist functions to accommodate the invading partly allogenic embryo.

1.14. Implantation

Successful implantation of a blastocyst is achieved by bidirectional crosstalk between the endometrium and embryo. Perturbation of this crosstalk, for example, caused by aberrant decidualization, compromises trophoblast invasion and may ultimately cause loss of pregnancy (Zhang et al., 2013). Traditionally, implantation was thought to be the result of active trophoblast invasion into a passive maternal decidua (Boyd 1980). Recent co-culture studies have brought a paradigm shift in the way we understand how stromal cells behave in the presence of a blastocyst. This study visualised the

invasive and migratory property of stromal cells, as the embryo was actively encapsulated by decidual stromal cells (Grewal et al., 2008, Teklenburg et al., 2010a).

Approximately, 2-8% of the endometrial epithelial cells are ciliated and display microvilli (Masterton et al., 1975). Upon contact with underlying stromal cells, the embryo expands beneath the epithelia and invades further. The stromal layer, though not decidualized, initiates differentiation by the presence of increasing progesterone levels (Gellersen and Brosens, 2014a).Trophoblast cells differentiate further to numerous primary syncytial elements and mononuclear cytotrophoblast. The epithelial cells cover the surface of the embryo following penetration (Aplin and Ruane, 2017). Key adhesion molecules such as mucins, integrins play a major role in the process of implantation. A receptive endometrium and the timing of implantation are critical for a successful implantation. A temporary period termed the 'window of implantation' ensures that a competent embryo is embedded into an optimal environment. Stromal cells and progenitors, upon differentiation, confer the endometrium with the ability to be both receptive and selective to a competent embryo. For successful implantation, the decidual cells must maintain control of the invasiveness of the embedding embryo to form the feto-maternal interface. Studies show that only decidualized human endometrial stromal cells encapsulate competent embryos and allow its expansion, compared to undifferentiated stromal cells. These differentiated cells, which express high levels of MMPs are motile and move around the embryo to encourage its expansion (Weimar et al. 2013).

Decidualization plays a vital role in conferring immunotolerance to the semiallogeneic embryo. Decidualized stromal cells modulate the local immune cell environment by increasing the influx of uterine NK cells, neutrophils and macrophages (Reiger et al., 2004). Animal studies show that the uterine dendritic cells (uDCs) are trapped by decidual stromal cells, preventing the migration of uDCs and identifying the embryo (Erlebacher 2013). Studies have shown that the decidual cells exhibit an initial

proinflammatory response, followed by an anti-inflammatory response with immunomodulatory properties. In decidua, uNK cells are suggested to suppress T-cells by apoptosis using galectin-1 (Blois et al. 2007).

Decidualized stromal cells are thought to act as a biosensor to selectively trigger tissue destruction when they encounter a developmentally compromised embryo, through some unknown mechanisms. Thus, decidualization not only creates a proinflammatory and anti-inflammatory response but also reactivates the inflammatory phenotype to prevent the embedding of an incompetent embryo (Salker et al. 2012).

The receptive state of the endometrium which is needed for successful implantation is acquired approximately six days after the postovulatory rise in progesterone and lasts around 2 to 4 days. This is known as the window of implantation and coincides with the spontaneous decidualization of the endometrium. In the absence of a fertilised egg, progesterone levels fall, leading to menstruation. The maternally controlled decidualization process in humans, which precedes menstruation, is considered to be an essential method of maintaining the reproductive fitness of mother (Haig. 2010). Progesterone signal withdrawal from decidualizing endometrium is a common signal for menstruation or menstrual-like shedding to discard the implanting incompetent embryo (Gellersen and Brosens, 2014a).

1.15 Menstruation

In the absence of implantation, the corpus luteum decays, progesterone levels decline, initiating a cascade of events in the functional endometrial layer that leads to tissue breakdown, menstrual shedding and scar-free repair (Jabbor et al. 2006). The concept of menstruation as an inflammatory event was proposed by Finn in 1986 as it shares features of inflammation (Finn, 1986). In a non-conception cycle, the inflammatory events before menstrual shedding is governed by the fall of ovarian steroid hormones, oestrogen and progesterone during the late secretory phase. The

fall in progesterone releases NF-κB from its inhibition by IκB which leads to induction of inflammatory gene networks which results in an influx of inflammatory cells. Interactions between inflammatory cells and decidualized stromal cells result in the release of a spectrum of pro-inflammatory mediators including chemokines, cytokines and prostaglandins. Pro-inflammatory factors and inflammatory cells lead to production and activation of a degradative enzyme cascade, particularly matrix metalloproteinases, ultimately resulting in the rapid breakdown of the extracellular matrix (Salamonsen, 2003, Salamonsen et al., 1999).

1.16 Relevance of hypoxia in menstruation

Declining levels of progesterone results in a cascade of molecular changes inducing myometrial contraction and vasoconstriction of spiral arterioles of the endometrium. These changes create a hypoxic environment in the functional layer similar to necrosis or ischemic injury (Markee, 1940). Transcription factor formed by HIF isoforms bind to hypoxia responsive elements (HRE) and regulate some physoxia responsive genes that are expressed in menses (Critchley et al., 2006, Maybin et al., 2011, Maybin et al., 2012).

Some contradictions exist in accepting that hypoxia plays a vital role in regulating tissue degradation and initializing the tissue repair process (Zhang and Salamonsen, 2002). In a xenograft model, endometrial functional layer explants were implanted in immunodeficient mice to determine whether physoxia plays a role in destruction or regeneration of endometrium upon hormone withdrawal or replenishment. The results showed that hormone withdrawal caused tissue destruction along with increased production of MMPs and TIMPs. However, there was no apparent change in partial oxygen pressure or HIF1α staining in the tissues. This study also analysed the oxygen level following hormone replenishment which found no evidence of physoxia (Coudyzer et al., 2013). However, a recent study with high throughput techniques showed that a spatial and temporal gradient of physoxia is necessary for

rapid tissue breakdown as well as for the regeneration of normal endometrial architecture (Cousins et al., 2016).

1.17 Angiogenesis in human endometrium

Amongst various physiological changes that occur during the menstrual cycle, there is profound angiogenesis in response to physoxia and sex steroid hormones. The major regulator of angiogenesis and remodelling is local physoxia environment. Reconstruction of the new vascular network and rapid vessel growth following menstrual destruction is regulated by oestrogen whereas progesterone regulates the vessel maturation. The tightly controlled angiogenesis is mediated by various pro- and anti- angiogenic factors such as vascular endothelial growth factor (VEGF), soluble VEGF receptor 1 (sVEGFR-1), angiopoietin (ANGPT), stromal cell-derived factor 1 (SDF-1/CXCL12), IL-8 and angiogenin (Okada et al., 2014).

Physoxia induces VEGF expression through physoxia inducible factor-1α (HIF-1α) (Lin et al., 2004). The effect of physoxia on VEGF production in endometrial stromal cells may have a physiological relevance in the process of menstruation, postmenstrual repair and angiogenesis (Tsuzuki et al., 2011). Oestrogen was reported to increase VEGF expression in endometrial stromal cell culture (Okada et al., 2010).

The maturation of newly formed blood vessels is induced by ANGPT1 associating with endothelial cells, pericytes and vascular smooth muscle cells. ANGPT2, an antagonist of ANGPT1, is involved in neovascularization along with VEGF. Increased ANGPT2/ANGPT1 ratio is associated with neovascularization. Physoxia reduces ANGPT1 expression whereas there is no change in the levels of ANGPT2. As physoxia increases VEGF expression, high ANGPT2/ANGPT1 ratio results in higher new blood vessel formation (Okada et al., 2014). Oestrogen also has similar effects to physoxia in the way it induces VEGF and attenuates ANGPT1, thus playing a role

in the reconstruction of vascular architecture of the endometrium (Tsuzuki et al., 2013).

1.18 Blood supply to the uterus

Ovarian and uterine arteries supply blood to the uterus. The uterine arteries arise from the anterior division of internal iliac artery and branch into arcuate arteries. These arcuate arteries further branch into radial arteries which penetrate the myometrium to provide blood to all layers of the endometrium. In the endometrium, radial arteries are modified into basal and spiral arteries that supply the functionalis layer. Basal arteries support the basalis layer of the endometrium, and these arteries do not respond to hormonal cues. In the absence of an implanting embryo, constriction of terminal arteries occurs, resulting in endometrial breakdown and shedding of the stromal and glandular compartments (Emedicine.medscape.com, 2017).

1.19 Spiral arteries

The radial arteries branch into spiral arteries upon reaching the myo-endometrial junction. Spiral arteries are separated from each other by a distance of 1-6 mm (Boyd and Hamilton, 1970). Before pregnancy, spiral arteries respond by growth and decidualization. The growth of spiral arteries is massive as it has to allow about 600 ml/minute of blood into the intravillous space (Figure 1.5). Research on spiral artery remodelling comparing normal and preeclampsia patients revealed that decidualization did not reach a myometrial layer that occurs in normal pregnancy. In a normal pregnancy, the mean diameter of myometrial spiral arteries in the placental bed was 500 µm whereas it was only 200 µm in cases of preeclampsia (Brosens et al., 2010, Brosens et al., 1972). Failure in physiological transformation is also considered as a cause for intrauterine growth restriction (IUGR) as there is a high vascular resistance that reduces the flow of blood to the intrauterine space (Brosens et al., 2010).

1.20 Perivascular niche

The initial perivascular niche was reported in hematopoietic stem cells because of their sinusoidal endothelium association both in bone marrow and spleen (Kiel et al., 2005). Perivascular localisation of hematopoietic stem cells (HSCs) within bone marrow is suggested as a reason for regulation of quiescence nature. Chondroitin Sulfate Proteoglycan 4 (CSPG4) coated arterioles are shown to maintain the quiescence nature, but their deletion induces HSCs cycling (Bourke et al., 2009, Kunisaki et al., 2013). Apart from these number of factors secreted by endothelial cells and MSCs (e.g. stem cell factor) within this perivascular environment is reported to be vital for maintaining the niche conditions. Plastic adherent non-hematopoietic perivascular cells capable of forming fibroblastic colony-forming units (CFU-F) (Friedenstein et al., 1970) are termed as mesenchymal stem cells (MSCs) (Caplan, 1991). With the use of flow cell sorting CD10, CD13, CD44, CD73, CD90, CD105 expressing mesenchymal stem cells were isolated from the perivascular cells (Oh and Nör, 2015). These cells exhibited potential to differentiate into mesenchymal lineages such as osteocytes, chondrocytes and adipocytes (Doherty et al., 1998, Farrington-Rock et al., 2004).

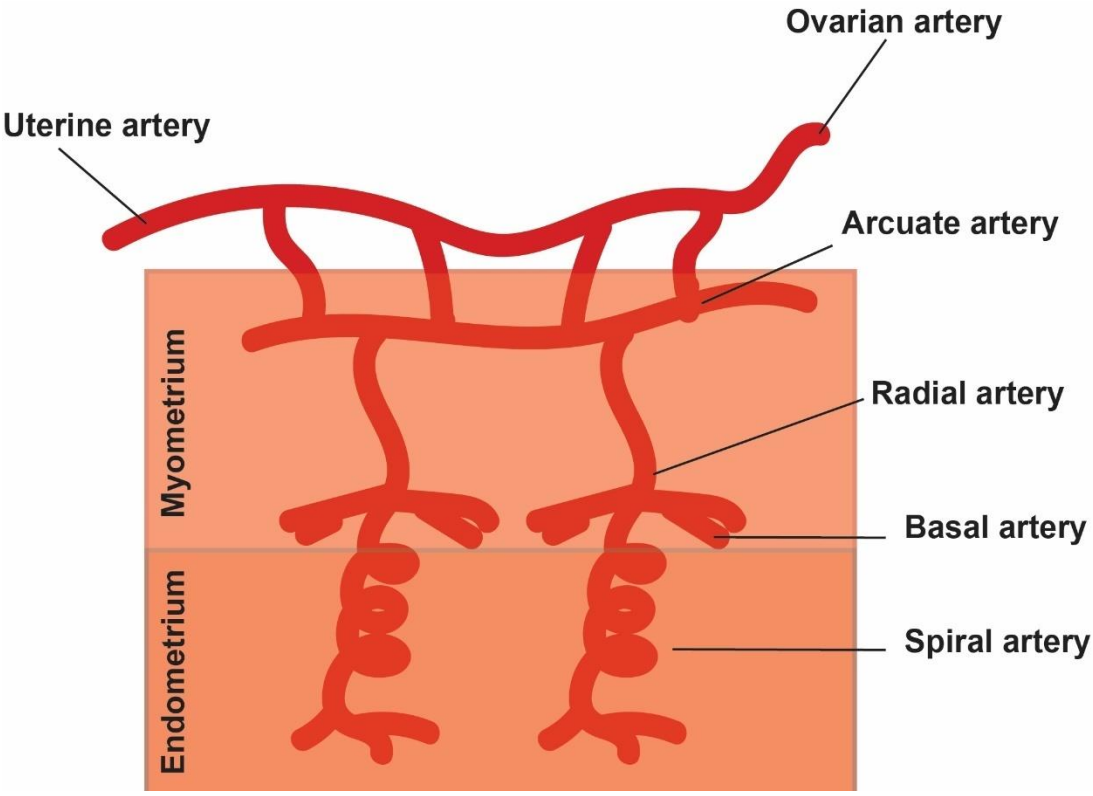

Figure 1.5. Vascular architecture within the uterus.

Uterine arteries branch into arcuate arteries and radial arteries which supply blood for the myometrial layer. Spiral arteries penetrate and supply blood to the functionalis layer of the endometrium. Figure adapted from (Robertson, 1976)

1.21 Stem cells

Regeneration following menstruation in each cycle is due to the presence of stem cells within the endometrium, a concept which was put forth four decades ago and was proven recently in 2007 (Gargett et al., 2007). Stem cells are initially involved in the embryological development and in due course help to maintain tissue homoeostasis throughout the lifetime. Stem cells are non-lineage specific, with potential plasticity with uni, multi -or pluri- potency. Asymmetric cell division results in one progenitor cell, which can differentiate into a particular lineage, and self-renewal of the stem cell (Figure 1.6). They reside in a microenvironment - 'niche' that provides support for the stem cells by cell-cell or cell-matrix interactions.

Embryonic stem cells and somatic stem cells are the two primary classifications of stem cells. Embryonic stem cells are embryonically derived, whereas somatic stem cells are undifferentiated stem cells amongst differentiated cells in adult tissues. Somatic stem cells comprise of mesenchymal and hematopoietic stem cells. Mesenchymal stem cells with a potential to differentiate into osteoblasts, adipocytes, chondrocytes and hematopoietic stem cells into blood lineage cells. There are some sources for mesenchymal stem cells including the umbilical cord, cord blood, bone marrow, and menstrual blood. Stem cells are usually quiescent unless there is a need to repair or regenerate when they migrate and replenish or recruit critical immune cells by chemokine/cytokine action. Immunomodulatory properties of mesenchymal stem cells are elicited in mice via nitric oxide, and in humans through some factors including indoleamine-2, 3-dioxygenase (IDO) and Leukaemia inhibitory factor (LIF) (Spaggiari et al...2010; Nasef et al. 2008). *In vivo* studies tracked the mesenchymal stem cell niche to be perivascularly located (Crisan et al. 2008, Hirschi et al. 1996).

As the embryo develops, pluripotential nature decreases thus giving rise to multipotent or unipotent somatic stem cells, which are found in most organs of the human body. The presence of stem cells within the endometrium was proposed four

decades ago based on the cyclical nature of the tissue. However, the actual evidence of stem cell existence in human endometrium was put forth in 2007 following *in vitro* and *in vivo* analysis (Gargett et al., 2007). This identified two types of stem cell populations within endometrium: epithelial stem cells (eESCs) and mesenchymal stem cells (MSCs).

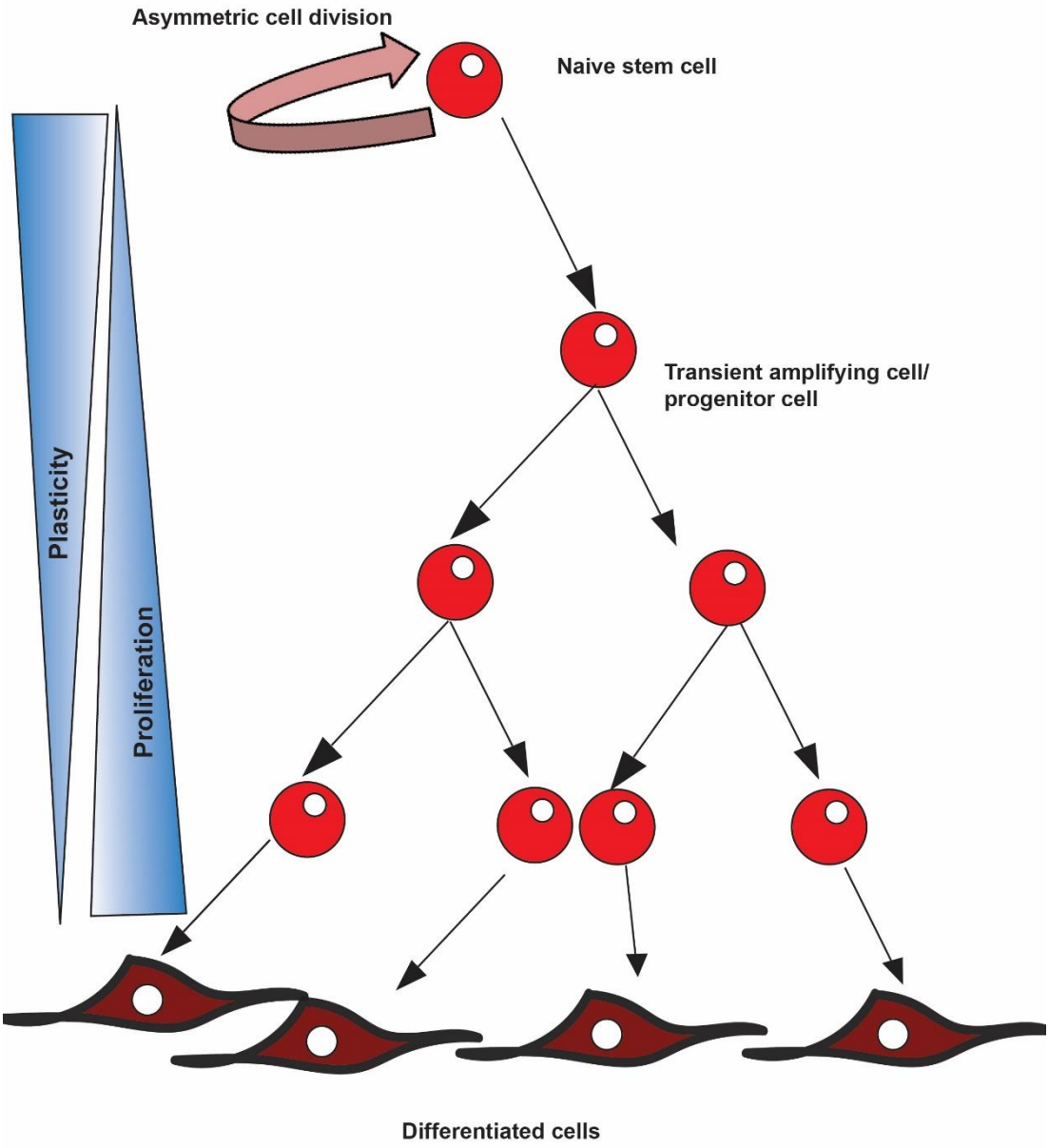

Figure 1.6. Cartoon showing stem cell undergoing asymmetric division.

Plasticity potential decreases as the division continues, whereas the differentiation potential increases. It is proposed that large colonies during CFU assay arise from stem cells and cells at progenitor stage give rise to smaller colonies.

1.22 Endometrial stem cells - Epithelial stem cells

The existence of ESCs came into light based on the fact that all endometrial glands are monoclonal in origin suggesting that they arise from a common progenitor cell. Following menstruation, a portion of glands remains in the basal layer of the endometrium. The epithelial cells from this layer re-epithelize the exposed surface and proliferate into the regenerated functionalis layer under the influence of oestrogen. Epithelial stem cells can form colony forming units (CFUs) and are proposed to be residing in the remaining glands following tissue destruction. ESCs constitute 0.08% of the endometrial epithelial cells and show immense self-renewal properties *in vitro* by undergoing 34 population doublings and the ability to differentiate into large glands in a 3D microenvironment. Stage-specific embryonic antigen 1 (SSEA-1, or CD15) is considered as a marker of ESCs as it is expressed strongly in the basal endometrial glands obtained via hysterectomy. SSEA-1 is strongly expressed in the glands of post-menopausal women and shows a similar gene profile as basal glands obtained from cycling women. In culture, SSEA-1+ ESCs have higher telomerase activity, longer telomeres and are proliferatively quiescent. When cultured in a 3D environment, SSEA-1+ ESCs form spheroids and further differentiated into spheres with polarised epithelium (Gargett et al., 2009). ESR1 and PR are expressed in lower levels in SSEA-1+ ESCs suggestive of their naïve state. Another marker, LGR5 (leucine-rich repeat containing G-protein-coupled receptor 5) is used to identify a rare epithelial stem cell population in the lower functionalis layer near the basalis layer. (Barker et al., 2007).

1.23 Endometrial stromal stem cells

1.23.1 Label-retaining cells (LRC)-evidence from mouse studies-

Stem cells remain quiescent, and this property is exploited to identify adult stem cells by labelling with 5-bromo- 2-deoxyuridine (BrdU). Since stem cells divide infrequently, they have the capacity to retain the dye for a longer period in comparison to actively proliferating cells. In mouse endometrium, the BrdU label is maintained by 6-9% of the stromal cells and 3% of epithelial cells (Cao et al., 2014). Oestrogen receptor (ER) was absent in the majority of these stromal and epithelial LRCs. However, 16% of the stromal LRC possessed ER. It can be speculated that these ER+ LRCs might be involved in the estrogen-induced proliferation of the endometrial during the proliferative phase. *In vitro*, although ER negative, epithelial and stromal LRCs proliferated in response to oestrogen (Cervello et al. 2007, Chan & Gargett, 2006).

1.23.2 Endometrial stromal stem cells - evidence from human studies

Human endometrium is remarkably resilient; it undergoes cyclical changes, proliferates, sheds and regenerates, coordinated by the rise and fall in circulating ovarian steroid hormones. The active cyclical regeneration and remodelling of endometrial glandular and stromal components imply the presence of stem/progenitor cells which are thought to play an indispensable role in tissue maintenance and function of the uterus (Teixeira et al. 2008).

Stem cells were identified in the endometrium by clonal, proliferative, differentiation potential (Table 1.2) and self-renewal properties (Gargett et al. 2004). The colony forming units from these endometrial stem cells were shown to give rise to two types of colonies, large colonies containing cells capable of self-renewal, and small colonies containing endometrial transit amplifying cells (eTAs) which acquire differentiation markers (Gargett et al. 2009).

Though there are some markers for isolation of mesenchymal stem cells, the W5C5 antibody was found to select for a perivascular population of cells enriched within both endometrium and bone marrow. W5C5 is an antibody for SUSD2, (Sushi domain containing 2) a type I integral membrane protein. These SUSD2 expressing $W5C5^+$ cells are found around the spiral arteries of the endometrium. W5C5 is a cognate antibody for SUSD2 (Sivasubramaniyan et al., 2013). These endometrial stem/progenitor cells can differentiate into neural lineages (Wolff et al., 2011), pancreatic lineages (Li et al., 2010, Santamaria et al., 2011) and hepatocytes (Santamaria et al. 2011). Cultured perivascular cells are enriched in for $CD146^+$ / $PDGFR\beta^+$ and are capable of only four of the mesenchymal lineages: adipogenic, myogenic, osteoblastic and chondroblastic. Platelet-derived growth factor subunit B (PDGFRB) gene encodes for CD140b, a tyrosine kinase receptor, and reported to be crucial for vascular development. CD146 is a class of cell adhesion molecule and a marker of endothelial cell lineage. Cells which lacked these markers were unable to differentiate (Gargett et al. 2009). A previous study indicated $W5C5^+$ population constitutes 7% of the total HESCs (Murakami et al. 2013).

A recent study reported that clonogenic cells derived from $CD140b^+$ and $CD146^+$ perivascular cells are found consistently throughout the menstrual cycle (Figure 1.7). These cells display high self-renewal capacity. This study also showed that the basalis layer contained a higher percentage of stem cells when compared to the functionalis (Shan et al., 2016).

Another study analysed the transcriptomic profile of $CD140b^+$ and $CD146^+$ perivascular cells and demonstrated that these stem cells are the precursors of the endometrial stromal cell population. This study also confirmed that endometrial stem cells are localised perivascularly around the smaller blood vessels (Spitzer et al., 2012). Various markers used for identifying stem cells within endometrium are listed in Table 1.3.

Further studies profiled the transcriptome of freshly isolated MSCs, non-MSC stromal cells using CD140b/CD146 markers with their short and long-term clonal counterparts. This analysis confirmed that MSCs are bonafide precursors of clonal non-perivascular HESCs and non-clonal HESCs. Based on this transcriptomic analysis, and to avoid confusion, I propose a simplified nomenclature (Table 1.4). Briefly, clonal cells isolated from the perivascular cell fraction are designated as eMSCs; eTA cells denote clonal cells isolated from the non-perivascular cell fraction; ePSCs indicate SUSD2+ perivascular population, the SUSD2- non-perivascular population is represented by EnSCs and HESCs represented mature progeny cells and suggested decidual stromal cells are further down the lineage of endometrial stromal cells when treated with progesterone (Barragan et al., 2016).

1.23.3 Side population (SP)

The side population (SP) phenotype is also used as a marker for stem cells. SP cells are capable of effluxing Hoechst 33342 due to the presence of ATP-binding cassette (ABCG2) transporter. Clonogenic endometrial cells are enriched with SP cells. In culture, isolated SP cells are slow growing, a characteristic feature of stem cells. Schwab and Gargett (2007), showed that these SP cells are found in proximity to those expressing platelet-derived growth factor receptor beta (PDGFRβ) and CD146. Moreover, SP cells are capable of regenerating into entire endometrium when transplanted into immunocompromised mice (Masuda et al. 2010). Another study established that these side population cells constitute 0.00-5.11% of the total cell population of a normal endometrium (Kato et al. 2007).

Table 1.2. Differentiation potential of endometrial stem/progenitor cells

Markers expressed	Differentiated into	Possible Therapeutic potential	Reference
CD146, CD105 and CD90	Smooth muscle cells (SMCs)	Bladder reconstruction	Shoae-Hassani et al... 2013; Gargett et al. 2009
PDGF-Rβ, CD146	Dopaminergic neurons	Parkinson's disease	Wolff et al... 2010
PDGFβ-R, CD146, CD90	Insulin-secreting cells-pancreatic beta cells	Diabetes	Santamaria et al. 2011
CD73, CD146, CD90	Hepatocyte-like cells	Liver-related disorders	Yang et al. 2014
PDGF-Rβ, CD146	Adipocytes, osteoblasts, chondrocytes		Wolff et al. 2007; Dimitrov et al. 2008; Gargett et al. 2009

Table 1.3. Different markers for endometrial stem/progenitor cells

Stem cell marker	Property	Reference
Low Hoechst 33342 fluorescence	DNA stain that specific for side population	(Masuda et al., 2012b)
BrdU	Label retaining cells	(Cervello et al., 2011)
CD146 and PDGFRβ	Pericyte markers; Fluorescence-activated cell sorting (FACS)	(Schwab et al., 2005)
SUSD2	Uses W5C5 antibody; Perivascular markers; MACS sorted	(Masuda et al., 2012b, Murakami et al., 2014)
LGR-5* and *SSEA-1	Endometrial epithelial progenitor cells	(Valentijn et al., 2013, Sun et al., 2009)

Table 1.4. Simplified nomenclature of subpopulations of HESCs

Simplified Terminology	Specific marker/s
Endometrial mesenchymal stem cells (eMSCs)	Clonal $SUSD2^{+}$
Endometrial transit amplifying cells (eTAs)	Clonal $SUSD2^{-}$
Endometrial pericytes (ePCs)	$SUSD2^{+}$
Endometrial stromal cells (EnSCs)	$SUSD2^{-}$
Endometrial pericytes (ePCs)	CD146 and Platelet-derived growth factor receptor β (PDGFRβ) co expression
MenSCs	CD146 and Platelet-derived growth factor receptor β (PDGFRβ) co-expression from menstrual blood

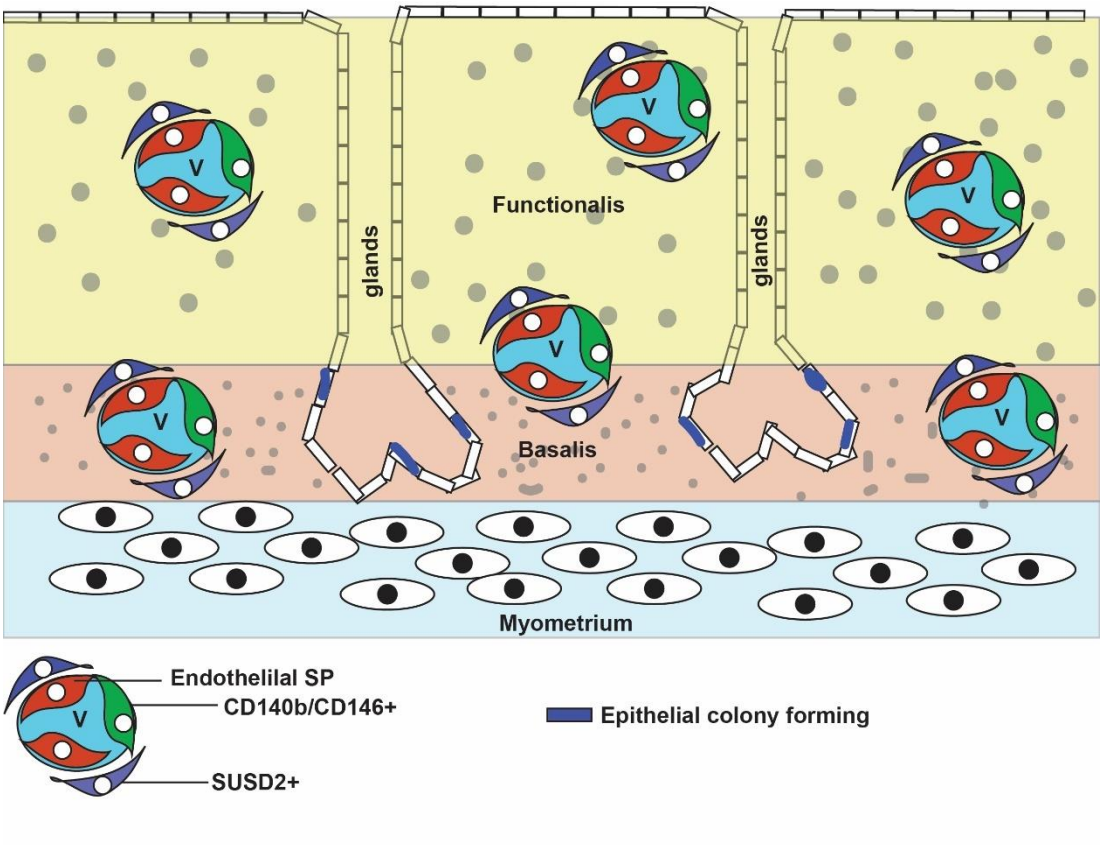

Figure 1.7. Endometrial perivascular cell localisation.

Epithelial cells with the ability to form colonies are proposed to be located at the base of the glands. SUSD2+ and CD140b/CD146+ pericytes are localized adjacent to the blood vessels (v). Side population (SP) cells express markers for endothelial as well as pericytic origin. Figure adapted from (Gurung et al., 2015).

1.24 Menstrual blood-derived stem cells (MenSCs)

CD146+/PDGFR-β+ cells have been isolated from menstrual blood (Schwab and Gargett, 2007), and are designated MenSCs. MenSCs also express MSC markers such as CD9, CD44, CD29, CD73, CD90 and CD105 along with pluripotency markers OCT-4, C-KIT and Stage-specific embryonic antigen-4 (SSEA-4) but lack expression of STRO-1, a marker which is expressed in perivascular cells isolated from endometrial biopsies. MenSCs undergo 68 passages when cultured *in vitro*, demonstrating its proliferative property. These cells showed tri-lineage differentiation potential of differentiating into osteoblasts, chondrocytes and adipocytes (Meng et al., 2007a, Musina et al., 2008, Patel et al., 2008). Menstrual blood perivascular cells produce high amounts of MMPs and factors promoting angiogenesis (Gargett et al., 2014). Menstrual blood perivascular cells showed varied differentiation potential by differentiating into cardiomyocytes, hepatocytes, and neural and pancreatic lineages from all three germ layers (Meng et al., 2007b, Patel et al., 2008). MenSCs are considered to be an easily available source for cardiac stem cell therapy (Bockeria et al., 2013).

1.25 Clonogenicity

A unique property of stem/progenitor cells is that they can form a clone from a single cell. Studies proved that the endometrial stromal stem/progenitor cells and epithelial cells could form colonies when seeded in a low cell concentration. The clonogenic capacity is lower in epithelial cells compared to stromal cells. Growth factors play a major role in the formation of colonies. Basic fibroblast growth factor (bFGF) is required for endometrial stromal progenitor cells to form colonies, whereas PDGFβ, tumour growth factor alpha (TGFα) and epidermal growth factor (EGF) are important in colony formation from epithelial progenitor cells (Chan et al., 2004).

Clonogenicity varies between epithelial and stromal progenitor cells during the various stages of the menstrual cycle. High clonogenicity was elicited by stromal progenitor

cells during proliferative phase, and by epithelial progenitor cells during the secretory phase. Chan et al. (2004) could show that within 15 days, 0.22% of endometrial epithelial cells and 1.25% of stromal cells were capable of forming individual colonies. Clonogenicity was higher in both ePCs and SP cells compared to EnSCs. ePCs also demonstrated differentiation potential like that of mesenchymal stem cells from any other source. The differentiation potential of clonogenic endometrial stromal stem/progenitor cells by inducing the cells into adipogenic lineage (Dimitrov et al., 2008). A study analysing endometrial tissue biopsies showed that clonogenic potential is enriched in ePCs population compared to EnSCs. Interestingly, the body mass index (BMI) negatively correlated with the proportion of ePCs, suggesting that the plasticity of the endometrium is compromised in obese individuals, which may potentially result in adverse pregnancy outcomes. (Murakami et al. 2013).

1.26 Decidualization effect on ePCs

Preliminary studies from our lab suggest that the decidual markers PRL and IGFBP1 are expressed at similar levels in both ePCs EnSCs. However, upon decidualization ePCs express a significantly different secretome when compared to EnSCs. Analysis of the cytokine profile at transcription levels shows that ePCs cells elicit a pro-inflammatory response on decidualization, in comparison to the anti-inflammatory responses mounted by EnSCs. Secretome analysis of conditioned medium from differentiating and non-differentiating ePCs show that decidualization induced perivascular cells to secrete high levels of immunomodulatory factors (Murakami et al., 2014).

1.27 Trophoblast invasion into decidua

In normal human pregnancy, the fetal trophoblast cells invade into the wall of the uterus in a complex and tightly controlled process. From tips of anchoring villi, cytotrophoblast cell columns are formed and a shell develops through which extravillous trophoblasts invade decidua. By the eighth week of gestation, interstitial cells from extravillous trophoblasts reach the superficial myometrium (Pijnenborg et al., 1981). In the uterus, the blood vessel diameter decreases sequentially as it progresses through myometrium and endometrium. Spiral arteries supply blood to the endometrial layer and span inner myometrium and for decidua in the case of a pregnant uterus. During pregnancy, placental bed spiral arteries undergo vascular remodelling from high resistance low flow vessels into low resistance high flow vessels (Whitley and Cartwright, 2010).

Perivascular cells which are terminally differentiated and are capable of switching between contractile and proliferative phenotype surround the spiral arteries in the endometrium (Kaplan-Albuquerque et al., 2005). The contractile phenotype is due to expression of contractile proteins such as α-actin, smooth muscle myosin heavy chain, calponin, and smooth muscle 22α. Perivascular cells do not proliferate, migrate or synthesise extracellular proteins (Owens et al., 2004). In response to changes in extracellular cues, perivascular cells adopt a more synthetic phenotype leading to proliferation and migration.

During spiral artery remodelling, a change in phenotype of perivascular cells might lead to increased sensitivity to apoptotic stimuli (Su et al., 2006) or increased migration (Newby, 2006). Defects in the phenotype switching might lead to altered vessel structure, loss of layered organisation of cells or migration away from lumen as evidenced in atherosclerosis (Newby, 2006).

Decidualization is associated with the influx of maternal natural killer cells that are present during spiral artery remodelling and eventually decrease during mid-gestation. In mice, it was shown that uNK cells are involved in spiral artery remodelling stimulated with interferon gamma (IFNγ) (Ashkar et al., 2000). In humans, it was demonstrated that uNK cells along with IFNγ produce TNFα, LIF, CSF-1and IL-8 (Ashkar and Croy, 2001, Hanna et al., 2006). IFNγ has been shown to antagonise decidual PRL expression (Christian et al., 2001).IFNγ can cause apoptosis as well as the proliferation of perivascular cells. IFNγ may prime the perivascular cells for death receptor-induced apoptosis (Bai et al., 2008). Interferon γ can increase TRAIL production in trophoblasts (Phillips et al., 1999). Immunohistochemical studies show that uNK cells and macrophages are localised at the site of spiral artery remodelling suggesting that immune cells may play a role in the reorganisation of vascular cells and smooth muscle layer separation (Smith et al., 2009). uNK cell also produces a myriad of angiogenic factors including VEGF, PLGF and Ang-2 (Hanna et al., 2006).A defect in vascular remodelling leading to uteroplacental hemodynamics has been considered to be a causative factor for pre-eclampsia (Hanna et al., 2006).

1.28 Endometrial scratch

Intentional injury to the endometrium of a woman wanting to conceive is termed as endometrial scratching. This insult to the endometrium is considered to increase the chance of an embryo implanting in turn increasing the chance of pregnancy. Various instruments are utilized to perform the biopsy. The most used instrument for performing endometrial scratch is a thin flexible plastic tube, 3mm wide, called a Pipelle catheter. The Pipelle is inserted through the cervix into the uterus and moved back and forth and rotated to cause some disruption. It is a simple, inexpensive outpatient procedure without anaesthetic and less time consuming.

1.29 Relevance of hypoxia in stem cell niche

The stem cell niche is defined as a specific anatomical compartment that includes cellular and acellular factors that combine both systemic and local cues to regulate the biology of stem cells (Jones and Wagers, 2008, Li and Xie, 2005). This specialised microenvironment is a combination of cells, blood vessels, matrix glycoproteins and in a 3D environment (Scadden, 2006). Stem cells' self-renewal and potential to differentiate is maintained by the interaction and communication with this microenvironment (Scadden, 2006).

The physiological oxygen concentration in the stem cell niche is maintained between 2-8% (Simon and Keith, 2008). The purpose of low oxygen concentration is to prevent the oxidative stress that is developed by reactive oxygen species during aerobic respiration (Busuttil et al., 2003). The oxygen concentration in the stem cell niche are as follows: hematopoietic niche: 1-6% O_2 (Grant and Root, 1947, Cipolleschi et al., 1993, Chow et al., 2001) ; mesenchymal niche: 2-8% O_2 (Kofoed et al., 1985, Harrison et al., 2002, Matsumoto et al., 2005) and neural stem cell niche: 1-8% O_2 (Dings et al., 1998, Erecińska and Silver, 2001).

As the physiological oxygen concentration in the stem cell niches is low, hypoxia could be termed as 'physoxia'. Though the laboratory oxygen concentration is termed normoxia, however it is rather high oxygen concentrations as opposed to physiological oxygen levels. So, I define this lab oxygen levels as 'hyperoxia'.

Below 5% O_2 concentration, glucose uptake and lactate production are high in MSCs compared to 21% O_2. Oxygen concentration over 5% O_2 concentration showed no difference compared to normal lab oxygen conditions. Oxygen consumption by stem cells was less in MSCs (Papandreou et al., 2006, Brown et al., 2007, James et al., 1995). Physoxia is shown to enhance proliferation in adipose-derived MSCs (Kakudo et al., 2015).

Mesenchymal stem cells located perivascularly exhibit low oxygen concentrations. Physoxia may serve as a stimulus to mobilise stem cells to sites of injury (Rosova et al., 2008). Studies show that MSCs cultured under physoxia showed low differentiation potential as the physoxia condition help in maintaining the quiescence state. MSCs cultured in hypoxic culture conditions show increased migratory phenotype than cells maintained in normal laboratory oxygen conditions (Rosova et al., 2008). Under physoxia, the Akt signaling pathway is activated and upregulates c-Met, a receptor for hepatocyte growth factor (HGF) (Rosova et al., 2008) which increases expression of VEGF, upregulates the phosphorylation of focal adhesion kinase (Lee et al., 2010) and increases the chemokine receptors such as CXCR4 and CX3CR1 expression (Hung et al., 2007). In several disease models, hypoxic or ischemic tissues are shown to produce cytokines and chemokines (Liu and McCullough, 2013). MSCs are responsive for recruitment of humoral factors to sites of injury, ischemia, and physoxia (Ceradini and Gurtner, 2005; Rafii and Lyden, 2003). Following recruitment, these cells respond to the ischemic site by expressing angiogenic factors (Potier et al., 2007). Murine MSCs cultured in glioma cell conditioned media under low oxygen condition formed capillary-like structures thus showing angiogenic phenotype (Annabi et al., 2003). In conclusion, low oxygen tension is certainly an important regulator in deciding the cell fate and in the maintenance of MSCs stemness.

1.30 Relevance in disorders

Stem/progenitor cells are involved in tissue homoeostasis and regeneration throughout the menstrual cycle, and studies are showing their relevance in endometrial disorders which may lead to pregnancy failure.

A recent pilot study involving endometrial tissues obtained by biopsies from obese women has shown that there is a deficiency of ePCs clonogenic cells compared to the normal weight controls. The clonogenic efficiency of both ePCs, as well as EnSCs,

reduces with an increase in the body mass index of the subjects. It was understood from this study that endometrium of obese women has lower plasticity and regenerative potential compared to women with normal body weight. There was an increase in numbers of pregnancy losses in these obese women (Murakami et al. 2013).

Endometrial stem cells can be a potential cause of endometriosis as recent evidence has shown the presence of stem cells in the menstrual blood (Ulrich et al. 2013). Endometriosis can cause pelvic pain and infertility. The incidence rate is 6-10% in all women, and 35-50% of these women suffer the consequence of pelvic pain and infertility. The cells isolated from endometriotic lesions are variable as few presented one type of cells whereas some had polyclonal cells. Another theory suggests an extrauterine origin of stem cells which cause endometriotic lesions. To prove this theory, Du and Taylor (2007) implanted wild type endometriotic implant in a hysterectomized LacZ expressing transgenic mouse. They could observe the LacZ expressing cells in the otherwise wild-type endometriotic lesions implant. Bone marrow derived stem cells which are thought to be involved in endometrial regeneration may also cause endometriotic lesions (Sasson et al. 2008).

In human reproduction, it is estimated that 30% of embryos are lost before implantation, 30% result in early pregnancy loss, and a further 10% of clinically recognised pregnancies. Thus, the loss of pregnancy and embryo wastage is much high in humans. (Rai and Regan, 2006)

In Europe, recurrent pregnancy loss (RPL) is defined as three or more consecutive miscarriages whereas in the United States of America it is defined as two or more losses (Quenby et al., 2002). On an average, 0.5-1% of couples will experience RPL (Tho et al., 1979). There are some factors which are associated with RPL, such as perturbation in anatomy and subclinical endocrine, immunological and clotting disorders (Li, 1998). However, unequivocal evidence for a causal link between

subclinical disorders and RPL is invariably lacking, and most, if not all, interventional trials have so far been unsuccessful (Quenby et al., 2002). Based on the emerging role for decidual cells in embryo biosensoring (Brosens and Gellersen, 2010), RPL has recently been attributed to impaired decidualization (Weimar et al., 2012, Salker et al., 2011a, Salker et al., 2010). Further, recent work from our lab has linked the aberrant decidual response in RPL patients to a deficiency in eMSCs and eTA cells, leading to heightened endometrial senescence and a blunted decidual response (Lucas et al., 2015). Further, the concept that lack of embryo quality control at implantation causes RPL is supported by epidemiological evidence that 40% of affected women are 'super fertile', defined by time-to-conception for each pregnancy of less than or equal to 3 cycles (Salker et al., 2010). RPL patients showed increased PROK-1 expression which promotes embryo-endometrial interaction and extended proinflammatory phenotype (Koot et al., 2012). Aberrant decidualization and poor endometrial quality control are thought to result in a prolonged 'window of implantation', which allows out-of-phase implantation in an unaccommodating uterine environment. However, the cumulative rate of successful pregnancies is high in RPL (Lucas et al., 2016a), indicating that embryo-endometrial interactions at implantation are intrinsically dynamic and may changes from cycle to cycle.

Table 1.5. Abnormal decidualization in reproductive pathologies

	Defect	Effect	References
Deregulated LIF	Infertile women	Indispensable for decidualization and implantation	(Stewart et al., 1992, Franasiak et al., 2014)
Aberrant decidualization	Endometriosis	Eutopic endometrium and ectopic lesions	(Aghajanova et al., 2010a, Aghajanova et al., 2010b, Klemmt et al., 2006, Aghajanova et al., 2009, Minici et al., 2008)
Irregular CpG methylation	Endometriosis	Increased methylated CpG sites in ectopic lesions-leading to blunted decidual response	(Dyson et al., 2014)
Refractoriness to progesterone treatment	Endometriosis	Very low gene expression alterations	
Disordered IL33-ST2L-sST2L axis	Recurrent pregnancy loss	Prolonged proinflammatory response during decidualization	(Salker et al., 2012a)
Reduced endometrial MSCs	Recurrent pregnancy loss	Abnormal decidualization	(Murakami et al., 2013b)

1.31 Hypothesis and Research aims

The extraordinary regenerative ability of the endometrium to adapt to changes, physiological or otherwise, depends on eMSCs with inexhaustible self-renewing and differentiation capacity. However, cyclic shedding, regeneration and rapid oestrogen-dependent growth also render the stromal compartment intrinsically heterogeneous. The imbalance between various stromal cell subpopulations (i.e. eMSCs, TA cells, ePCs and mature EnSCs) may account for the aberrant decidual response associated with either implantation failure or early pregnancy loss. Based on this changing endometrial paradigm, the specific research aims of my project were:

1. *To assess the robustness of clonal eMSCs /eTAs cell isolation from mid-luteal biopsies*. Colony-forming unit-fibroblast (CFU-F) activity was measured in freshly isolated HESCs from mid-luteal biopsies from RPL and infertile patients. To assess the robustness of this assay - a prerequisite for investigating the role of endometrial progenitor cells in reproductive failure - experiments were performed in triplicate and repeated in two consecutive cycles. This experimental design also enabled assessment of the impact of tissue injury (i.e. biopsy) on endometrial clonal cell populations in different patient groups.

2. *To validate the link between aberrant decidualization and reproductive failure*. Although impaired decidual responses in primary HESCs has been associated with major reproductive disorders, such as endometriosis and RPL, there is as yet no evidence that the same mechanism underpins sporadic implantation failure. To test this possibility, a detailed study was carried out to examine the decidual response in primary cultures established from biopsies obtained from unselected patients in the cycle before a successful or failed IVF treatment cycle.

3. *In-depth characterization of eMSCs / ePCs cells*. Accumulating evidence indicates that eMSCs residing in the perivascular niche are the primary drivers of endometrial regeneration and, by extension, its function. However, these cells are only partly characterised. My analysis fortuitously revealed that eMSCs have a unique ability to form 3D organoids that resemble the gross triangular morphology of the endometrium. These new organoids termed endometrial regenerative bodies (ERBs), provide potentially a new model to study physiological and pathological implantation events.

Chapter 2

2 Materials and Methods

2.1 Materials

Material	Supplier	Catalog number
β-estradiol	Sigma-Aldrich	E2758
8-Bromoadenosine 3',5'- cAMP	Sigma-Aldrich	B7880
6-well plates	Corning	3598
12-well plates	Corning	3513
24-well plates	Corning	3506
96 well plates	Corning	3599
Antibiotic/Antimycotic (Penicillin, streptomycin, amphotericin B)	Gibco	15240-062
BSA	Sigma-Aldrich	A2153
Cell Recovery Solution	Corning	354253
Cell scrapers	Corning	3010
Cell strainer, 40 µm	Fisher Scientific	22363547
Charcoal Sigma-Aldrich	Sigma-Aldrich	C9157
Chloroform	VWR	100034Q
Cleaning agent for removing RNase (RNAzap)	Sigma-Aldrich	R2020
Collagenase	Sigma-Aldrich	C9891
Cytokeratin 18 antibody Mouse	Abcam	AB668
DNAse I for tissue digestion	Roche	11284932001

DPX mounting medium	Leica	3808600E
Dulbecco's modified Eagle medium (DMEM)/F12 nutrient (Ham) (1:1) medium with red-phenol	Gibco	11039-021
Eosin Y	Sigma-Aldrich	HT110132
Epidermal growth factor (EGF)	Peprotech	AF100-15
Eppendorf tubes 1.5 ml	Starlab	E1415-1500
Eppendorf tubes 0.6 ml	Starlab	E1405-0600
Ethanol	Fisher Chemical	E/0650DF/17
Falcon tubes 14 ml	Greiner Bio-one	188261
Falcon tubes 50 ml	Greiner Bio-one	227270
Fetal bovine serum (FBS)	Gibco	10500-64
FGF10	Peprotech	100-26
Filter paper	Whatman	3001-861
Formaldehyde, 4%	VWR	9713
Glass-bottom Petri-dishes	Mat Tek	P35GCol-1.5-10-C
Glass Pasteur pipettes	Fisher Scientific	1156-6963
Glass slides	Thermo Scientific	BS7011/2
Gloves	Kimtech Science	90626
Glycogen	Invitrogen	10814-010

Insulin	Sigma-Aldrich	91077C
Isopropanol (2-Propanol)	Sigma-Aldrich	24137
L-glutamine	Gibco	25030-024
Matrigel	Corning	354230
Medroxiprogesterone 17-acetate	Sigma-Aldrich	M1629
Nuclease-free water	Ambion	AM9932
Parafilm	Bemis	PM-996
PBS	Fisher Scientific	102092521X-PBS
PCR plates FAST	Applied Biosystems	4346906
PCR plates non-FAST	Applied Biosystems	N8010560
PCR plate sealing film	Excel Scientific	TS-RT2-100
Pipette tips 10 µl	Alpha Laboratories	ZP1010S
Pipette tips 40 µl	Alpha Laboratories	ZP1204S
Pipette tips 100 µl	Alpha Laboratories	ZP1200S
Pipette tips 300 µl	Alpha Laboratories	ZS3300S
RTCA plates (E-plate)	ACEA Biosciences	05469813001
Scalpels	Swann-Morton	0501
Secondary antibody, anti-mouse Alexa-Fluor 488	Molecular Probes	A11088
Secondary antibody, anti-mouse Alexa-Fluor	Molecular Probes	A21424
Serological pipettes 5 ml	Greiner Bio-one	606 180

Serological pipettes 10 ml	Greiner Bio-one	607 180
Serological pipettes 25 ml	Greiner Bio-one	760 180
STAT-60	AMS Biotechnology	CS-502
Sybr green	Applied Biosystems	4367659
Syringes 20 ml	BD Plastipak	300613
Syringes 50 ml	BD Plastipak	300865
Syringe filter	Sartorius Stedim Biotech	16534-K
Vectashield mounting medium with DAPI	Vector Laboratories	H-1500
Vimentin antibody Mouse	Cell Signalling	3390
Medium 200	Thermo Fisher Scientific	M-200-500
Low Serum Growth Supplement (LSGS)	Thermo Fisher Scientific	S00310
Luna Automated BF Cell Counter	Labtech	L10001
Cell Counting Slides	Labtech	L12002
MiniMACS Separation columns	Miltenyi	130-042-201
MiniMACS Separator	Miltenyi	130-042-102
XF24 FluxPak	Seahorse Bioscience	100867-100
XF Base Medium	Seahorse Bioscience	102353-100
Oligomycin	Sigma-Aldrich	O4876-5MG
Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone	Sigma-Aldrich	C2920-10MG
Rotenone	Sigma-Aldrich	R8875-1G

Seahorse XF24 Cell Culture Microplates	Agilent Technologies	
Seahorse XF24 FluxPaks	Agilent Technologies	

2.2 Methods

2.2.1 Endometrial tissue biopsy processing

An endometrial biopsy was obtained using a Pipelle endometrial sampler (CCD, Paris). Biopsies were transferred to the laboratory in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% dextran-coated charcoal-treated fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Invitrogen, Paisley, UK). The tissues were washed twice in DMEM/F12, finely minced, and enzymatically digested with collagenase (134 U/ml; Sigma-Aldrich, Gillingham, UK) and deoxyribonuclease type 1 (156 U/ml; Roche, Burgess Hill, UK) for one h at 37^{o}C. The digestion mixture was shaken every 20 minutes to enhance the process. After centrifugation at 125 $\times$ g for 5 min, the pellet, consisting of dispersed endometrial cells, was re-suspended in FBS containing 20% dimethyl sulfoxide (DMSO), frozen at an approximate rate of -1°C / min and the following day, stored in liquid nitrogen.

2.2.2 SUSD2 magnetic separation

Following previously described protocol (Masuda et al., 2012b, Murakami et al., 2013a), with several modifications, human endometrial stromal cells (HESCs) were isolated as single-cell suspensions from mid-luteal biopsies. Samples were washed in DMEM/F-12 medium (Invitrogen), thoroughly minced, and enzymatically digested with collagenase (0.5mg/ml; Sigma-Aldrich) and deoxyribonuclease (DNase) type I (0.1 mg/ ml; Roche) for 1hour at 37°C in CO_2 incubator. The dissociated cells were filtered through a 40 µm cell strainer (Fisher Scientific). Stromal cells and blood cells, present as a single-cell suspension, passed through the cell strainer to remove the undigested fragments, mostly comprising of glandular clumps. Stromal single-cell

suspensions were layered over Ficoll-Paque PLUS (GE Healthcare) and centrifuged to remove erythrocytes. The medium/Ficoll-Paque PLUS interface, containing stromal cells, was carefully aspirated, washed with DMEM/F-12 Medium, twice. It was then subjected to magnetic bead separation to isolate ePCs and EnSCs as described previously. Briefly, freshly isolated HESC suspensions (up to $1x10^6$ cells/100µl of Magnetic Bead buffer consisting of 0.5% BSA in PBS) were incubated with phycoerythrin (PE) conjugated anti-human W5C5 antibody (5µl/$1x10^6$ cells; BioLegend) on ice for 20 minutes. Cell suspensions (up to $1x10^6$ cells/80 µl of Magnetic Bead buffer) were then incubated with anti-PE-magnetic-activated cell sorting Micro Beads (20 µl/$1x10^6$ cells; Miltenyi Biotec) on ice for 20 minutes. Cell suspensions (up to $1x10^6$ cells/500 µl of Magnetic Bead buffer) were applied onto MS columns (Miltenyi Biotec) in a magnetic field, followed by washing with 500 µl of Magnetic Bead buffer three times. Although W5C5 cells passed through the column, magnetically labelled ePCs were retained on the column. The columns were removed from the magnetic field, and ePCs cells were flushed with 1 ml of Magnetic Bead buffer.

2.2.3 RNA sequencing

Total RNA from freshly magnetically isolated ePCs and EnSCs cells were extracted using RNA STAT-60 (AMS Biotechnology) following the manufacturer's protocol, including DNase treatment (Invitrogen). RNA quality was analysed using Agilent 2100 Bioanalyzer (Agilent Technologies) and assessed with the Eukaryotic Total RNA Nano program according to the manufacturer's instructions. RNA integrity number score was made sure that for all samples were at least 9. Transcriptomic alignments were identified with bowtie-0.12.8 (Langmead et al., 2009), samtools-0.1.18, and tophat-2.0.4 (Trapnell et al., 2009) against the University of California Santa Cruz hg19 reference transcriptome from the Illumina iGenomes resource.

Gene counts were estimated using HTSeq-0.5.3p3(http://www-huber.embl.de/users/anders/HTSeq/).Transcripts per million were calculated as recently described (Wagner et al., 2012). Count data from the Top Hat-HTSeq pipeline were analysed using three different methods for differential expression detection, i.e., DESeq, baySeq, and edgeR (Robinson et al., 2010, Anders and Huber, 2010, Hardcastle and Kelly, 2010). Gene transcript abundances between ePCs and EnSCs were considered significantly different if the false discovery rate value (baySeq and edgeR) or adjusted P value (DESeq) was < .01. Differentially expressed genes were retained if they were detected by at least two of the methods used.

2.2.4 Primer designing

Sequences were obtained from the PubMed Human Genome database (https://www.ncbi.nlm.nih.gov/gene/). Primers were designed using Primer-Blast and Universal Probe Library Assay Design Center from Roche. Primers were designed were checked whether they followed the following requirements: Melting temperature (Tm) was made sure to be between 58.0°C and 59.9°C. Tm was made to differ between forward and reverse primer by greater than 1˚C. Total amplicon length was set to between 75 and 110 base pairs. At the 3’ end of the primer, of the last five bases, two bases should be either G or C. There is no more than four of the same base consecutively. Primer length was set to be between 18-24 bases. Primers are required to be exon spanning. Melting temperature (Tm) is calculated with the formula Tm = 69.3 + (41(GC/L)) - (650/L), where GC is the number of G and C bases in the primer and L is the number of nucleotides in the primer. Designed primers were cross-referenced using the Primer 3 Output Programme.

2.2.5 Primer efficiency

Primers were optimised to determine efficiencies. Forward and reverse primers were used at 300nM in a total volume of 19µl in an SYBR Green master mix and loaded

onto a 96-well plate. 1µl of pooled cDNA or 1µl nuclease free water was added per well in triplicate. The amplified product of the triplicate well combined, mixed with loading dye and ran on a 1% agarose gel, which ran for approximately 50 minutes at 100V. The purified product was excised from the gel using Qiagen Gel Extraction Kit (as described below) and cDNA concentration measured. Purified cDNA was serially diluted between 100pg/µl to 10ag/µl in 1/10 dilution factor providing eight dilutions. The serial dilutions were amplified using the appropriate primers and an SYBR Green Master Mix and Ct Values measured. The log of the concentration of cDNA was plotted against average Ct values. To calculate primer efficiencies, the following calculation was used.

$$\text{Primer efficiency} = 10^{\frac{-1}{\text{the gradient of the line}}}$$

2.2.6 RNA extraction

To minimise risks of RNA degradation, RNase-free plastic-ware and nuclease free water was used throughout, and the surfaces were cleaned with RNase ZAP. Total RNA was extracted from cells and tissues using STAT-60 reagent- a monophasic solution of phenol and guanidine isothiocyanate, which maintains RNA integrity and disrupt other cellular components. 400µl of reagent RNA Stat-60 reagent was added per well in a 6 well plate ensuring all cells were covered. Cells were scraped thoroughly using a Corning cell scraper and transferred to pre-chilled RNase-free 1.5ml Eppendorf tubes**,** placed on ice. Cold chloroform (neat) in a 20% volume of initial STAT-60 was added to the solution and mixed well by vortexing. This solution was allowed to stand at room temperature for 5 minutes and was transferred to dry ice. Samples were stored at -80°C. Samples were defrosted on ice and centrifuged at 12,000 x g at 4°C for 30 minutes to separate the sample into an aqueous and an organic phase. RNA remains exclusively in the colourless upper aqueous phase. The aqueous phase was carefully transferred into 50% volume of cold isopropanol(neat),

incubated at room temperature for 10 minutes to precipitate the RNA. RNA was pelleted by centrifugation at 12,000 x g at 4°C for 15 minutes, washed twice with 1ml 70% cold ethanol and air-dried for 2 minutes. The pellet was dissolved in an appropriate volume of nuclease-free water. RNA concentration and quality were assessed by Nanodrop. Satisfactory values were considered equal to or greater than 1.80 on the 260/280 absorbance scale, indicating pure RNA without any protein contamination. Samples were stored at -80°C or cDNA synthesis was carried out.

2.2.7 cDNA synthesis

For cDNA synthesis, I used QuantiTech Reverse Transcription Kit (Qiagen). All reagents were thawed on ice, mixed and centrifuged briefly to prevent any concentration gradients. gDNA wipeout buffer(7x) in a volume of 2 µl was added to 1µg of template RNA made up to a total volume of 14µl with RNase-free water, to remove any traces of genomic DNA. The samples were incubated at 42°C for 2 minutes and placed immediately on ice. A reverse transcription master mix was prepared to a volume of 10% greater than that required. Per reaction, 1µl of Quantiscript Reverse Transcriptase was added to 4µl 5x Quantiscript RT Buffer, along with 1µl RT Primer Mix. This was then added to the 14µl template RNA, reactions were mixed and stored on ice. Minus RT controls were also used in which 1µl nuclease free water replaced the 1µl of Quantiscript Reverse Transcriptase. All other stages were identical. Reactions were incubated at 42°C for 30 minutes, then inactivated by incubation at 95˚C for 3 minutes. cDNA samples were diluted with 30µl nuclease free water to give a final volume of 50µl.

2.2.8 Gene expression analysis by qRT-PCR

Genes of interest were amplified using SYBR Green detection reagent. Reactions were carried out on a 96-well plate in a total volume of 20µl. Primers were used at a concentration of 300nM and a 50:50 ratio between forward and reverse primers. 1µl

of cDNA template was added to the well and set up in triplicate for technical replicate. Non-template controls in which 1µl nuclease free water replaced the cDNA were also used. The optical plate was sealed with an optical cover, briefly centrifuged to remove air bubbles and placed in the qRT-PCR machine. Thermocycling conditions were as follows: 1) 50°C for 2 mins 2) 95°C for 10 mins 3) 95°C for 15 seconds 4) 60°C for 1 min Dissociation curves were also run to determine that the amplified products were specific, and that SYBR Green I fluorescence is a direct measure of accumulation of the product of interest.

2.2.9 Secretome analysis

Conditioned media collected from decidualized and undifferentiated EnSC cultures as well as embryo droplets were randomized and assayed in duplicate for 45 cytokines, chemokines and growth factors [BDNF, EGF, Eotaxin (CCL11), FGF-2 (FGF basic), GM-CSF, CXCL1 (GROα), HGF, IFNγ, IFNα, IL-1RA, IL-1β , IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8 (IL-8), IL-9, IL-10, IL-12 p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, CXCL10 (IP-10), LIF, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), βNGF, PDGF-BB, PLGF, CCL5 (RANTES), SCF, CXCL12 (SDF1α), TNFα, LTA (TNFβ), VEGF-A, VEGF-D] using a multiplex suspension bead immunoassay (Ebioscience), according to the manufacturer's protocol and as described previously 43, but with some modifications. Briefly, 50 µL of conditioned media was mixed with 50 µL of antibody-conjugated, magnetic beads in a 96 DropArray plate (Curiox Biosystems, Singapore) and rotated at 450 rpm for 120 min at 25°C while protected from light. Beads were internally dyed with different concentrations of two spectrally distinct fluorophores and covalently conjugated to antibodies against the 45 cytokines, chemokines and growth factor. The plate was washed three times with wash buffer (PBS, 0.05% Tween-20) on the LT210 Washing Station (Curiox) before adding 25 µL of the secondary antibody and rotating at 450 rpm for 30 min at 25°C protected from light. Subsequently, the plate was washed three times with wash buffer, and 10 µL of

streptavidin-phycoerythrin added and rotated at 450 rpm for 30 min at 25°C protected from light. The plate was again washed thrice with a wash buffer; 60 µL of reading buffer was then added, and the samples read using the Bio-Plex Luminex 200 (BioRad). The beads are classified by the red classification laser (635 nm) into its distinct sets, while a green reporter laser (532 nm) excites the phycoerythrin, a fluorescent reporter tag bound to the detection antibody. Quantitation of the 45 analytes in each sample was then be determined by extrapolation to a 6-point standard curve as the amount of fluorescence detected by the reporter laser is proportional to the amount of target present in the sample where data analysis of experimental data was carried out using five-parameter logistic regression modelling 47. Calibrations and validations were performed before runs and on a monthly basis respectively. Twenty-two factors [FGF-2 (FGF basic), GM-CSF, IFNγ, IFNα, IL-1RA, IL-1β, IL-1α, IL-2, IL-5, IL-7, IL-9, IL-10, IL-15, IL-17A, IL-21, IL-22, IL-23, IL-31, PDGF-BB, PLGF, SCF, LTA (TNFβ)] with measurements missing from >50% of samples were excluded.

2.2.10 Human embryo culture

The ethics committee of the Institutional Review Board of the University Hospital (UZ Brussel, Vrije Universiteit Brussel) and the Belgian Federal Ethical Committee for Scientific Research on Human Embryos approved this study and reviewed and approved the informed consent form. All experiments were performed in accordance with relevant guidelines and regulations. Vitrified day 5 blastocysts, which became available for research with written informed consent and after the legally determined storage period of five years, were warmed using the Vitrification Thaw Kit (Vit Kit-Thaw; IrvineScientific) and left to recover for 3 hours before morphological scored by an experienced independent clinical embryologist (according to Gardner and Schoolcraft criteria) (Table S2) 44,45. For assisted hatching, the zona pellucida was removed by pronase treatment (1 mg/ml; Sigma) for 10 min at 37°C. The resulting

zona-free blastocysts were washed 3 times in human tubal fluid (HTF) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (IVF Basics) medium containing albumin (CAF-DCF, Brussels, Belgium). Blastocysts were left to recover for 3 hours from pronase treatment and scored again on morphology (data not shown). Good quality late day 5 blastocysts were allocated randomly to pooled culture supernatant of undifferentiated EnSCs and cells decidualized for 2 or 8 days. The blastocysts were incubated undisturbed for 24 hours at 37°C in 5% carbon dioxide and 6 % oxygen. Day 6 blastocysts were morphologically evaluated, and growth rates (0, 1 or 2) determined, reflecting the degree of development based on expansion and morphology scores of inner cell mass (ICM) and trophectoderm (TE). A growth rate of '0' denotes that the embryo was degraded or failed to develop further; '1' indicates moderately or significantly expanded embryo with a "C"-score for the TE and/or ICM; and significantly expanded embryos with ≥ "B"-score for the TE and/or ICM were scored '2' (Table S2). The microdroplets were stored individually at -80°C for multiplex secretome analysis.

2.2.11 Real-time analysis

Real-time quantitative polymerase chain reaction was used to determine mRNA abundance, indicative of gene expression, using the ABI PRISM 7500 Sequence Detection System. An SYBR Green based assay was used in which a fluorescent signal is emitted once SYBR Green is incorporated into double-stranded DNA. Therefore, as the PCR progresses, higher quantities of double-stranded DNA accumulated and is measured at each cycle, thus allowing DNA concentrations to be quantified and assigned a Ct (cycle threshold) value. Ct values are defined as the number of cycles required for the fluorescent signal to cross a given threshold (i.e. exceeds background level). The analysis was carried out using the Delta Delta Ct Method, by which comparisons between the Ct values of the samples of interest with a control or normalizer such as a non-treated sample. The Ct values of both the

normalizer and the samples of interest are adjusted to the housekeeping gene L19. This results in a fold change value indicating the relative fold change of expression between the sample of interest and the normalizer.

2.2.12 Cell migration and proliferation-real time analysis

A Real-Time Cell Analyser (RTCA) DP xCELLigence instrument, was utilised with specialised microtiter culture plates containing an interdigitated gold microelectrode for assessing cell proliferation and migration. The principle in which xCELLigence works is that cell contact with the electrode increased the impedance across these gold arrays and reported as an arbitrary 'cell index' value as an indication of confluency and adherence. ePCs and EnSCs cells were seeded into 16-well E-plates at a density of 10,000 cells per well and cultured in DMEM supplemented with 10% FBS until confluent. The RTCA DP instrument was placed at 37°C in a humidified environment with 5% CO_2. Individual wells within the E-plate were referenced immediately and monitored first every 15 min for 3 hours and then hourly for 2-3 days. Changes in cell index were captured and analysed using the RTCA Software v1.2 supplied with the instrument.

2.2.13 Gel contraction measurement

For measuring the contractibility force, cells were cultured up to 90% confluence. The media for this assay was prepared by following the formulations as mentioned below (contraction media):

10X DMEM	135 µL
0.5M NaHCO3	118 µL
1M NaOH	13.5µL
L-Glutamine	13.5µL
P/S	13.5µL
FCS	67.5µL

Water	58.6mL

The cells were trypsinized and resuspended in the contraction media. The number of cells was enumerated using automated Luna cell counter. The cells were centrifuged and resuspended in collagen containing media which was prepared as follows:

Collagen Type I	675 µL
1M NaOH	12.6µL
Cells 10^6/mL	105 µL

The cells in collagen gel (1.5 ml) was pipetted into 6-well plates. This step was carried on ice to prevent the gelation of collagen. The plates were incubated in incubator till the gel was set. DMEM with 5% FBS (1.5ml) was added to the solid gel to feed the cells within the gel. The following day, the gel was dislodged from the plate using a gauge needle. The plate with floating gel was incubated further, and the media was changed to DMEM with 1% FBS after 8 hours. The gels were passed on to our collaborators in engineering department where the gel contraction per single cell was measured using a depth sensing indentation device.

2.2.14 Measurement of oxygen consumption rate

A day before the experiment, Flux Pak was hydrated by adding 1 ml of XF calibrant solution to the bottom plate. Care was taken not to scratch or damage the bottom of the electrodes. The Flux Pak was incubated in the 37^{o}C, no CO_2 incubator, overnight. XF Media was prepared with 200 ml of seahorse DMEM media by adding 0.9 of glucose (25mM) and 2ml of sodium pyruvate (100mM). pH was adjusted to 7.4. The drugs were prepared from stock for the following concentrations: (i)Oligomycin: 10 mM;(ii) Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP):10 mM (iii)Rotenone: 20 mM. Each drug was prepared in 4 ml volume in 10x the

concentration so that when mixed with the media it achieves the expected concentration. The XF seahorse instrument was switched on and was allowed to run overnight to maintain the temperature. On the day of the experiment, XF media was filtered using a syringe filter. DMEM media from the XF cell culture plate was removed, and 650 ul of XF media was added to each well-containing cells (20,000 cells/well), incubated at 37oC with no CO2 for 1 hour. The drugs were loaded to ports of the Fluxpak in the following order. A: Oligomycin: 75µl; B: FCCP: 83 µl; C: Rotenone: 93 µl. While the cells were in incubation, the software was programmed, and calibration step was carried out using Fluxpak with drugs. The program was run; the output was saved and analysed.

2.2.15 Colony forming unit assay

Freshly isolated stromal cells after Ficoll gradient separation and freshly isolated ePCs and EnSCs were seeded at a clonal density of 50 cells/cm^2 in a fibronectin coated 60mm dish or 6-well plate in culture medium containing basic fibroblast growth factor (bFGF) (10 ng/ml) (Merck Millipore, Watford, UK). Half media change was carried out on day 7, 10 and 12. The cells were either stained or passaged on day 15. The staining was done using hematoxylin. Clusters of ≥ 50 cells were counted, and the CE was determined from the formula:

$$\textbf{CE (\%)} = \frac{\textbf{number of colonies}}{\textbf{number of cells seeded}} \times \textbf{100.}$$

2.2.16 Physoxia induction

Physoxia was induced using Modular Incubator Chamber (Billups - Rothenberg Inc. California) and a cylinder containing 3% oxygen. The chamber was charged every day with 3% oxygen for 4 minutes at 20 bar pressure. The tightly sealed chamber containing the plates were maintained at 37°C with 5% CO_2 along with the control

cells without physoxia. Each condition was carried out in triplicates. At the end of 10-day incubation, the cells were treated for further experiments or harvested for RNA.

2.2.17 Differentiation

For decidualization experiments, colonies from ePCs and EnSCs were placed in phenol red-free 2% DCC-FBS supplemented DMEM-F12 overnight, and hormonal treatments were carried out the following day. For standard decidualization treatment, eMSCs and eTAs were treated in phenol red-free DMEM/F12 containing 2% DCC-FBS with 0.5mM 8-bromo-cAMP in combination with and 1µM medroxyprogesterone acetate. All experimental treatments were carried out in a reverse time course up to eight days.

2.2.18 *In vitro* angiogenesis assay

Aliquots of frozen Corning Matrigel (Fisher Scientific) was placed on ice, and a micropipette tip box was kept in the freezer to prevent the matrix formation while dispensing. Chilled Matrigel (50 µL) was added to each well and was incubated in 37^{o}C for 1 hour. After incubation, 5x10^{4} cells harvested from eMSCs and eTAs were added on top of the Matrigel in 200 µl volume of Medium 200 containing Low Serum Growth Supplement (LSGS). The cells were incubated in 37^{o}C overnight. Following day, wells were imaged using phase contrast microscope with 4x objective. The images were quantified for the area covered by either cells or tubes using ImageJ thresholding method.

2.2.19 ERB formation

While carrying out *in-vitro* angiogenesis assay, I fortuitously observed the formation of tissue structures (ERBs) on the surface of Matrigel. This protocol was designed and optimised for ERB formation.

ePCs and EnSCs were subjected to CFU-A assay to enrich eMSCs and eTAs and was allowed to grow for 12 days. On day 12, colonies were washed with sterile PBS, trypsinized and enumerated using automated Luna cell counter. The cells were resuspended in Media 200 which was supplemented with LSGS supplement.

Aliquoted Matrigel which was stored in -20^{o}C was thawed on ice for 13-15 minutes. Once Matrigel is liquefied, 50 µl was pipetted into a well of 96 well plate. 10^5 MSCs or TAs were added to the Matrigel and media was made up to 200 µl. The plate was then incubated at 37^{o}C with 5% CO_2. Following day, plates were assessed for ERB formation.

2.2.20 Time-lapse imaging

The formation of ERB was recorded for 48 hours for every 10 minutes using an Olympus microscope. The temperature control system and CO_2 were turned on a day before the experiment to equilibrate the setup and to maintain the conditions similar to the incubator. The set up involved a glass bottom dish with a clonal ring which formed a similar surface area of a 96-well plate. The same method for ERB formation was followed.

2.2.21 ERB co-culture

ERBs were allowed to form from MSCs following the protocol mentioned earlier. Human endometrial epithelial cells were cultured from frozen in a T25 flask were harvested and counted using automated Luna cell counter. 10^4 epithelial cells were added to the preformed ERB and incubated for three days. After three days, ERBs were fixed using 4% Formalin.

2.2.22 ERB paraffin embedding/sectioning

The ERBs were fixed using 100 µl formalin for 30 minutes and were transferred from Matrigel to 0.6 ml Eppendorf tube caps using a gauge needle and bent glass pipette.

1% agarose made with sterile PBS was added on to the ERB to fill the cap. The agarose disc, once set, was transferred into a Cell path system II hex cassette for processing overnight using Leica ASP200 S Fully Enclosed Tissue Processor. The processed agarose pad within the cassette was then set in paraffin wax and kept at 4^{o}C to form a block. The paraffin wax was placed on ice for at least half an hour to prevent wax from shredding while sectioning.

Paraffin sections were done using a microtome to 5 µm thickness and allowed to float in a water bath at 40 ^{o}C and then placed onto glass slides. The excess wax on the slide was removed by placing the slides in the oven at 65 ^{o}C.

The slides were rehydrated by a transferring across series of baths of xylene, 100% and 70% isopropanol and then rinsed in distilled water. Then slides were placed into citrate buffer (pH 6.0) and pressurised in a Pickcell unit for 2 hours and allowed to stand overnight. The slides were then gently washed with distilled water, and the tissue section was marked using a hydrophobic pen.

Slides were then blocked with 1% BSA for 1 hour at 4^{o}C and then incubated in primary antibody (1:200) overnight at 4^{o}C. The following day, slides were washed with 1% BSA for 20 minutes at 4^{o}C on rolling stage. Slides were incubated with secondary antibody for 1 hour at the same concentration as the primary antibody. Slides were then washed with 0.2% TBS-T for 30 minutes on a shaker. Slides were mounted using Vector hard set 4',6-diamidino-2-phenylindole (DAPI) and covered with a coverslip. Slides were allowed to dry and imaged by confocal and spinning disk microscopy.

2.2.23 Effect of agitation

To check the effect of agitation, preformed ERBs and preformed ERBs with epithelial cells were transferred into a new 96-well plate with organoid media and was agitated using an orbital plate shaker at 250 rpm for over two days.

2.2.24 ERB decidualization

ERBs were formed in the presence of angiogenic medium (Medium 200 supplemented with LSGS) and was left undisturbed for 48 hours. After 48 hours, the angiogenic media was withdrawn and was incubated in DMEM supplemented with 2% DCC for 1 hour. Following that, MPA and cAMP were added to the low serum media and was maintained for 0,2,4,6,8 days. After six days, the ERBs were fixed with formalin and stained with hematoxylin and eosin. The rest of the ERBs were maintained in low serum media supplemented with cAMP and MPA for eight days, following a reverse time course, RNA was harvested using STAT-60.

2.2.25 ERB physoxia vs. hyperoxia culture

Previously I found that oxygen concentration has a significant effect on some colonies formed and size of the colonies, I wanted to analyse whether it has some effect on the formation of ERBs. CFU-A was carried out in physoxia and hyperoxia using freshly isolated ePCs. The cells were cultured up to ten days and trypsinized using trypsin-EDTA. The number of cells was enumerated using automated Luna cell counter. 10^5 MSC cells were seeded in Matrigel in the presence of angiogenic media.

The cells were exposed to different oxygen concentrations in the following way:

Physoxia eMSCs ⟶ Physoxia for ERB formation

Physoxia eMSCs ⟶ Hyperoxia for ERB formation

Hyperoxia eMSCs ⟶ Physoxia for ERB formation

Hyperoxia eMSCs ⟶ Hyperoxia for ERB formation

The dishes were checked for ERBs the following day and imaged using phase contrast microscope using 4x objective. The ERBs were then measured using ImageJ.

2.2.26 Statistical analysis

GraphPad Prism 6 (GraphPad Software Inc.) was used for statistical analyses. Data were checked for normal distribution using Kolmogorov-Smirnov test. Unpaired or paired t-test was performed, as appropriate, to determine statistical significance between groups for normally distributed data. Mann-Whitney U test was used for non-normally distributed data. For comparing more than three groups, the data were analysed using One-way Analysis of Variance (ANOVA), followed by t-test with Bonferroni adjustment for pairwise comparisons. $P < 0.05$ was considered significant. Hierarchical clustering using Euclidean distance (MeV version 4.9.0) was performed on the cytokine profiles after the normalisation of significantly differential factors by first centring the data to the median and scaling it by division with the standard deviation. Secretome data was further analysed by partial least squares regression (PLSR) modelling (Unscrambler X version 10.1) after the normalisation of data by first centring the data to the median and scaling it by division with the standard deviation. Full cross-validation was applied in PLSR to increase model performance and for the calculation of coefficient regression values. The Chi-square test was used to determine differences in embryo growth rates.

Chapter 3

3 Assessment of the robustness of clonal EnSCs isolated from mid-luteal biopsies.

3.1 Introduction

Several studies reported a positive effect of endometrial injury during secretory phase on implantation in women suffering recurrent implantation failure following IVF treatment (Barash et al., 2003, Raziel et al., 2007, El-Toukhy et al., 2012, Potdar et al., 2012, Karimzadeh et al., 2009, Narvekar et al., 2010, Gnainsky et al., 2010, Almog et al., 2010). How endometrial trauma or injury improves implantation and clinical pregnancy rates in subsequent cycles are unclear at present. Notably, tissue injury was first reported to trigger decidualization in the guinea pig over a hundred years ago (Loeb, 1907). In fact, trauma has been used as a means to induce decidualization in various animal models, including mice (Bany, 2013). Trauma can be caused by intraluminal needle scratch, running a thread or, less traumatic, infusion of sesame oil through the uterine lumen (Croy et al., 2013).

In clinical setting, endometrial injury/biopsy/trauma is termed as 'endometrial scratch'. Endometrial scratch is a routine and easy procedure that is being carried out in implantation clinic prior to IVF for improving the chance of embryo implantation (Nastri et al., 2013).Endometrial scratching is carried out using Pipelle biopsy. This intentional damage to endometrium was reported to increase the chance of pregnancy in ART (Barash et al., 2003). Though the mechanism of how scratch improves the pregnancy is not explored, there are speculations of how it might work: (i) the scratch in previous cycle might induce decidualization thus increasing the chance of pregnancy (Li and Hao, 2009, Garris and Garris, 2003); (ii) might increase the secretion of factors that aid in implantation (Gnainsky et al., 2010); (iii) scratch might alter the asynchronous endometrium (Li and Hao, 2009).

The impact of tissue injury triggered by a biopsy, commonly referred to as an 'endometrial scratch', on endometrial stem cell populations has not yet been investigated. However, there are insights from other tissues. For example, a recent study identified two populations of quiescent stem cells in muscle: G_0 and G_{Alert} cells (Rodgers et al., 2014). Characterization of G_{Alert} cells revealed that these cells are in an intermediate state between quiescence and activation, i.e. poised to proliferate in respond to environmental stimuli like tissue damage without a need for an activation stage (Rodgers et al., 2014).

Wound healing following injury depends on coordinated interplay between various cells, growth factors and extracellular matrix proteins (Wu and Chen, 2014). In the case of vascular injury, cytokines released by vascular endothelium and activated platelets alter the stem cell niche and mobilise hematopoietic stem cells by providing a signalling gradient enabling homing to the site of injury. A similar mechanism might operate in the endometrium following physiological (e.g. menstruation and parturition), pathological (e.g. miscarriage) or iatrogenic tissue injury (Youn et al., 2011).

Puck and Marcus first reported clonogenic assay when clones formed from single mammalian cell when seeded in a suitable medium (Puck and Marcus, 1956). Following this, various in vitro techniques were developed for various kinds of stem cells. Eventually clonogenic assay became an alternate method for demonstrating its functional properties. Clonogenic property was used as a tool to identify adult stem cells based its ability to form colonies when seeded in low cellular density (Franken et al., 2006). Both epithelial and stromal cells isolated from endometrial basalis have the potential to form colonies when subjected to colony forming unit-fibroblast (CFU-F) assay. A small population of human endometrial epithelial cells (0.22–0.52%) and stromal cells (1.25%) was shown to possess clonogenic activity in vitro (Chan et al., 2004). Both epithelial and stromal cells initiated two forms of colonies (i) small, loosely

arranged and (ii) large, densely packed. Most of the colonies were small, and near about 37% of epithelial colonies were large and in case of stromal cells only 1 in 60 colonies were large (Chan et al., 2004). It is postulated that larger colonies are formed by committed progenitor cells with higher proliferation capability whereas the smaller colonies are formed by less proliferative transient amplifying cells. Also the stem cells with ability to form larger colonies are capable of entering the cell cycle when placed in a favourable *in vitro* environment, as stem cells have large nucleus-cytoplasmic ratio (Radley et al., 1999).

The role of endometrial tissue injury in improving implantation and life birth rates has been the subject of considerable controversy. Interestingly, there are yet no studies on the impact of endometrial injury on life-birth rates in recurrent miscarriage patients.

In this chapter, I used CFU-F to quantify clonal (eMSCs / TA cells) in the endometrium following biopsy-induced endometrial injury. The clonogenic assays were performed in patients who attended the Implantation Research Clinic with a history of early pregnancy loss and nulliparous patients undergoing IVF treatment. The recurrent miscarriage was defined as 3 or more consecutive miscarriages and 'sporadic miscarriage' as less than three miscarriages.

3.2 Results

3.2.1 Study cohort: demographic variables

Luteal-phase endometrial biopsies were obtained in 2 consecutive cycles from eighteen women. The miscarriage group consisted 10 women, 7 of which suffered recurrent miscarriages and 3 sporadic miscarriages. The infertile group was heterogeneous, consisted of 8 infertile patients receiving IVF treatment for unexplained infertility, male factor and Polycystic ovary syndrome (PCOS). Demographic and clinical details are summarised in Table 3.1. The median (± SEM) age of the miscarriage and infertile groups was 35 (± 1.5) years and 37 (±1.4) years respective ($P > 0.05$). The median body mass index (BMI) in the miscarriage and infertile groups were comparable (BMI: 23± 1.7 and 20 ± 0.8, respectively), although the data were incomplete.

3.2.2 Miscarriage versus Infertile: Colony-forming unit-fibroblast (CFU-F)

Primary endometrial stromal cells were isolated from all biopsies as described in Chapter 2 and frozen at -80°C until further analysis. To assess the CFU-F, frozen endometrial stromal suspensions from paired biopsies were thawed and subjected to colony forming assays. To evaluate the robustness of the assay, each assay was performed in triplicate. Figure 3.1 and Figure 3.2 show colony formation in the 1st and 2nd biopsy from miscarriage and infertile groups, respectively.

The average (± SEM) CFU-F activity of stromal cells in the 1st biopsy was significantly lower in the miscarriage group (2.06 ± 0.9%) when compared to infertile group (5.36 ± 0.97%), $P < 0.05$ (Figure 3.3 A). However, in the 2nd biopsy endometrial CFU-F activity in miscarriage group was comparable to the infertile group: 5.23 ± 1.1% versus 3.23 ± 0.7%, respectively (P > 0.05; Figure 3.3 B).

Comparison of CFU-F activity of stromal cells between 1st and 2nd biopsy in each patient group showed that tissue injury significantly increased CFU-F activity in the miscarriage group ($P < 0.05$), but not in the infertile group (Figure 3.4 A, B). Notably, increased number of colonies was observed in 6 out of 7 recurrent miscarriage patients, and 2 out 3 patients who suffered sporadic miscarriages in the miscarriage group. CFU-F activity in miscarriage group was 2.06 ± 0.9 % and 5.23 ± 1.1 % (mean ± SEM, n=10) for the 1st and 2nd biopsy, respectively. In infertile group, CFU-F activity was 5.36 ± 0.97 % and 3.23 ± 0.7 % (mean ± SEM, n=8) for the 1st and 2nd biopsy, respectively.

There is a significant increase in the fold induction of CFU-F activity in miscarriage group compared to infertility group ($P < 0.05$) **(Figure 3.5).** The data suggest that the CFU-F which was low following first biopsy increases following the endometrial injury in the miscarriage group whereas there was no difference in the case of the infertile group.

Table 3.1. Characteristics of women from whom the biopsies were obtained.

ID	Age	Live-births	1st trim loss	BMI	Phase	Other
1.1	29	0	5	17	S	Recurrent miscarriage
1.2					S	
2.1	42	0	5	24	S	Recurrent miscarriage
2.2					S	
3.1	34	0	4	26	S	Recurrent miscarriage
3.2					S	
4.1	43	0	7	36	S	Recurrent miscarriage
4.2					S	
5.1	31	0	4	23	S	Recurrent miscarriage
5.2					S	
6.1	31	0	20	21	S	Recurrent miscarriage
6.2					S	
7.1	36	0	9	22	S	Recurrent miscarriage
7.2					S	
8.1	39	0	1	25	S	Sporadic Miscarriage
8.2					S	
9.1	36	Un	2	23	S	Sporadic Miscarriage
9.2					S	
10.1	32	0	1	Un	S	Sporadic Miscarriage
10.2					S	
11.1	37	0	0	Un	S	Infertility
11.2					S	
12.1	37	0	0	Un	S	Infertility
12.2					S	
13.1	41	0	0	22	S	Infertility
13.2					S	
14.1	37	0	0	24	S	Infertility
14.2					S	
15.1	29	0	0	20	S	Infertility
15.2					S	
16.1	39	0	0	Un	S	Infertility
16.2					S	
17.1	34	0	0	20	S	Infertility
17.2					S	
18.1	41	0	0	20	S	Infertility
18.2					S	

*S stands for secretory phase. Un stands for unknown.

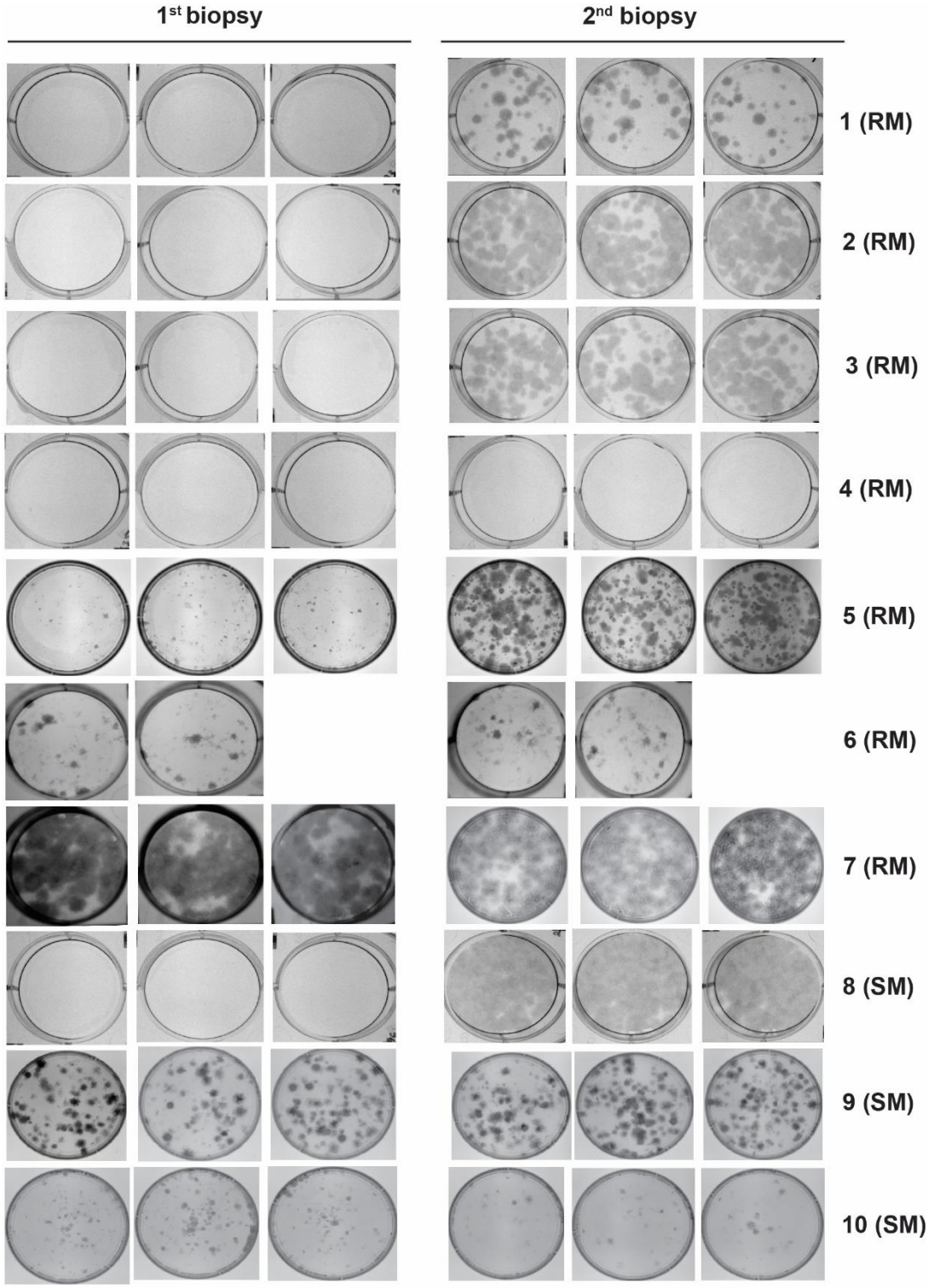

Figure 3.1. CFU-F of paired biopsies from miscarriage patients.

The abundance of clonogenic cells in the follow-up biopsies in women with miscarriages compared to the initial biopsies. Representative images of CFU-F assay plates in triplicates obtained after subjecting primary EnSCs to colony-forming assays. RM denotes recurrent miscarriage and SM for sporadic miscarriage.

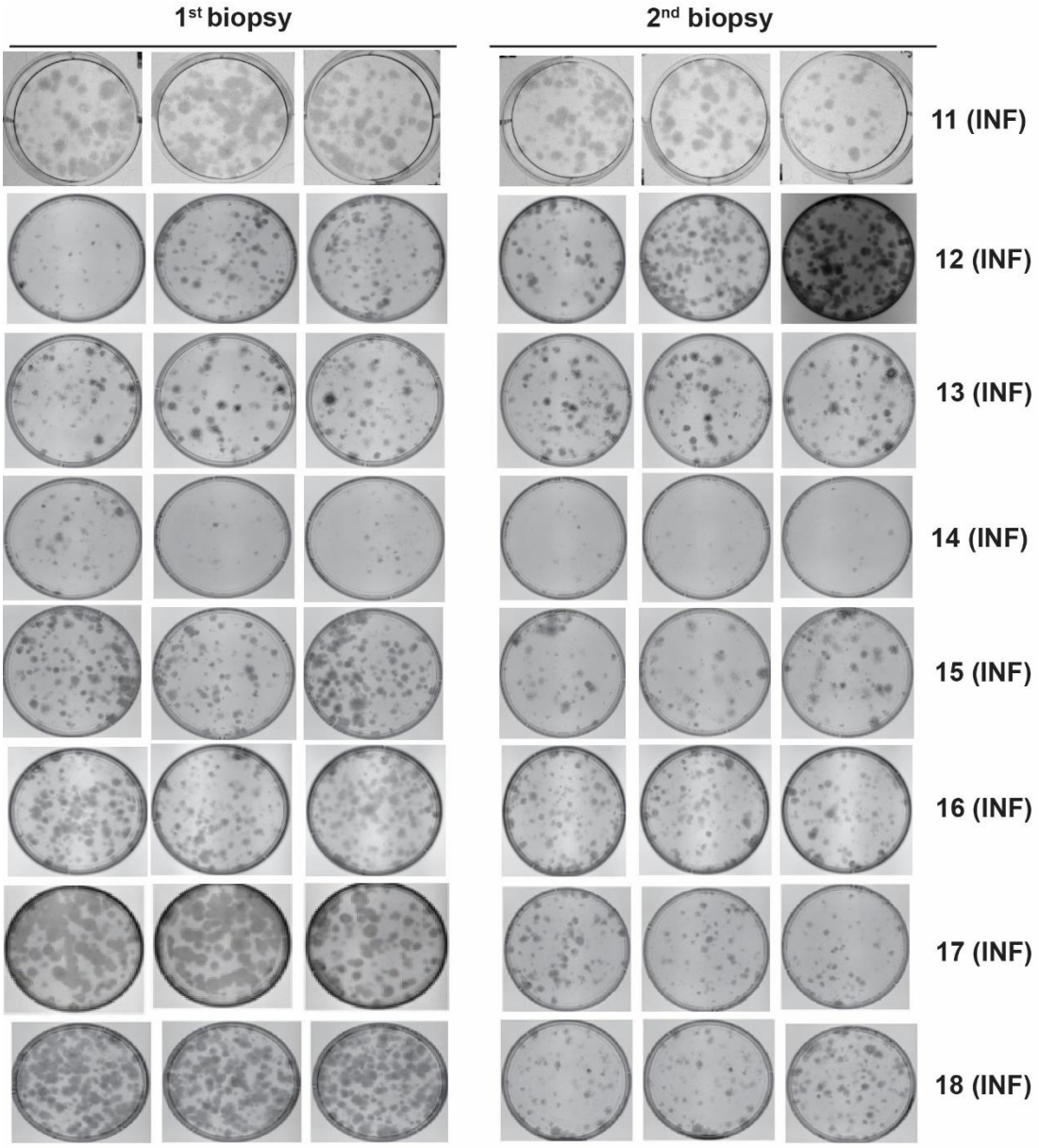

Figure 3.2. CFU-F of paired biopsies from the infertile group.

Reduction of clonogenic cells in the follow-up biopsies in women with other infertility (INF) issues, compared to the initial biopsies. Representative images of CFU-F assay plates in triplicates obtained after subjecting primary EnSCs to CFU-F assay.

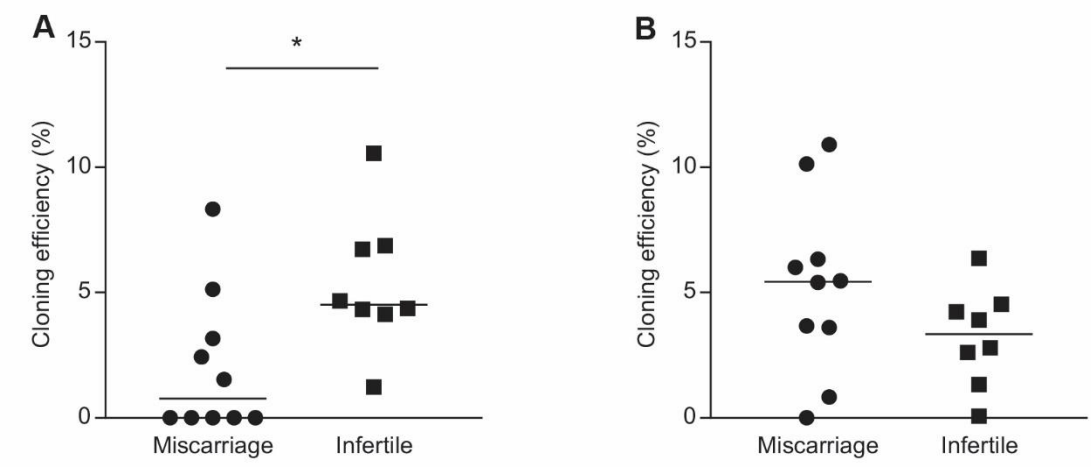

Figure 3.3. Cloning efficiency comparing miscarriage and infertility groups

Colonies with > 50 cells were counted, and cloning efficiency (CE) calculated. A. CE between the first biopsies in women with miscarriage and infertile group. B. Cloning efficiency of second biopsies in women from miscarriage and infertile group. * indicates $P < 0.05$. Data represent mean ± median.

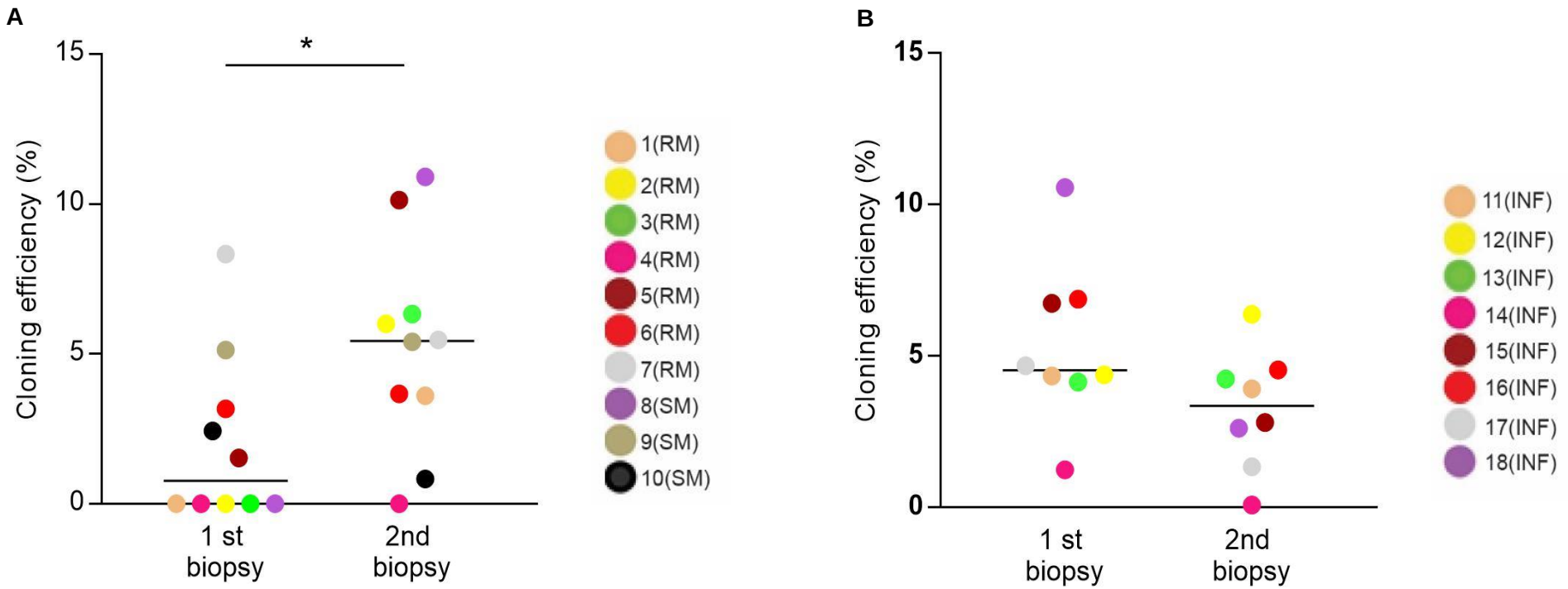

Figure 3.4. Cloning efficiency comparing first and second biopsies within each group

Colonies with > 50 cells were counted, and cloning efficiency (CE) calculated. A. Comparison of CE between the first biopsy and the second biopsy in women who had miscarriages. B. Cloning efficiency of first and second biopsy in the infertile group. * indicates $P < 0.05$. Data represent mean ± median. Each sample pair is coded with the same colour

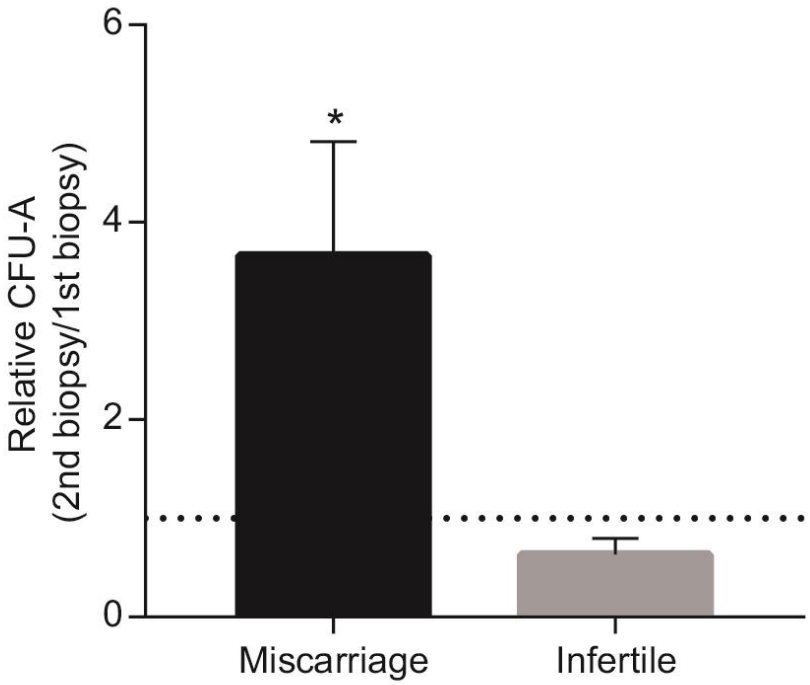

Figure 3.5. Relative CFU-F in miscarriage and infertile group.

Colonies with > 50 cells were counted, and cloning efficiency (CE) was calculated. Fold change in CE was calculated between the first biopsy and the second biopsy from miscarriage and infertile group. * indicates $P < 0.05$. Data represent mean ± SEM.

3.3 Discussion

Tissue repair and regeneration following injury or trauma is a coordinated process that involves proliferation, migration, and recruitment of stem cells and subsequent differentiation into tissue-specific lineage (Li and Clevers, 2010). Stem cells reside in their niche to replenish and repair tissues in response to insult. This is achieved by their remarkable proliferative capacity. Due to the asymmetric property of stem cells, a subset remains in a quiescent state for a prolonged period. The quiescent state of stem cells is, therefore, the key to maintaining cellular and tissue homoeostasis (Li and Clevers, 2010).

Quiescent stem cells are characterised by reduced RNA content (Fukada et al., 2007), lack of expression of proliferation markers (Gerdes et al., 1983), and the ability to retain DNA labels (Conboy et al., 2007). Maintenance of these cells in a quiescent state is regulated at epigenetic, transcriptional and post-transcriptional level. Although in a quiescent state, stem cells sense environmental stimuli and have the ability for re-entering the cell cycle and proliferate (Cheung and Rando, 2013). The quiescent state is regulated by mechanisms that are energetically favourable in transforming low metabolic stem cells to rapid global responses needed for activation (Cheung and Rando, 2013).

The role of endometrial tissue injury in improving implantation and life birth rates has been the subject of considerable controversy. A systemic review and meta-analysis of 7 randomised clinical trials (RCT) with 907 participants reported that endometrial injury improved clinical pregnancy rates significantly ($P < 0.00001$) in women with recurrent implantation failure (Coughlan et al., 2015). The clinical pregnancy rate considerably improved following an injury to the endometrium in women with recurrent implantation failure (Confidence interval (CI) 95% CI 2.04–6.01, $P < 0.00001$) and suggestive of benefit in women embarking on their first cycle but without significance (95% CI 0.93–1.86, $P = 0.12$). Another RCT compared the implantation rates in 115

participants who randomised into an endometrial biopsy or control group. Following biopsy, the implantation rate in the biopsy group was 10.9% compared to 3.38% in the control group. Clinical pregnancy rate was 27.1% in biopsy group whereas it was 8.9% in the control group. (Karimzadeh et al., 2009).

The absence of a discernible impact of the biopsy of CFU-A of isolated HESCs from infertile subjects was disappointing. However, it should be noted that this patient group was clinically heterogeneous. Hence, it would be interesting to assess the impact of tissue injury on clonal endometrial cell populations in patients with recurrent implantation failure.

The mechanism underpinning the effects of endometrial injury remains poorly understood. A similar mechanism for stem cell recruitment following vascular injury might be taking place in human endometrium following biopsy-induced trauma. Upon vascular injury, hematopoietic stem cells (HSCs) increase in the circulation in clinical and experimental conditions. HSCs need to be mobilised from their bone marrow niche to the peripheral circulation to be recruited to the site of injury. This is achieved by the release of soluble factors at the injury site that forms a concentration gradient. G-coupled protein receptor CXCR4 and its ligand CXCL12 are identified as important factors that maintain HSCs within the bone marrow and also for recruitment at the site of injury (Rocha and Broxmeyer, 2010). High levels of CXCL12 retain HSCs within the bone marrow, and low levels lead to mobilisation. The CXCL12 level is significantly increased in the blood flow following an injury (Petit et al., 2002), and expression is partly controlled by oxygen concentration (Ceradini et al., 2004). Adhesion molecules within the bone marrow such as Vascular cell adhesion protein 1 (VCAM-1) (Papayannopoulou and Nakamoto, 1993), hyaluronic acid (Avigdor et al., 2004) and P- and E- selectins (Xia et al., 2004) are cleaved, releasing HSCs adhered to the niche within the bone marrow. Neutrophil elastases and cathepsin G cleave VCAM-1 which releases the adhered stem cells, permitting their mobilisation to the

blood stream (Lévesque et al., 2001). Platelets, the first line in wound repair, also play a role in recruiting HSCs and MSCs to the injured site. MSCs which are recruited to injured sites are capable of differentiating into endothelial cells and releasing proangiogenic factors (Langer et al., 2006).

CXCR4 and CXCL12 or stromal-derived factor 1 (SDF1) interaction, which is vital for homing of stem cells to the injured site, is enhanced by nitric oxide, interactions of Jagged/Notch and MCP1/CCR2, growth factors such as VEGF and G-CSF.

Following injury, endothelial nitric oxide (eNOS) increases the expression of SDF-1 through the cGMP-dependent mechanism, where nitric oxide acts as a gaseous signalling molecule, which eventually causes the recruitment of stem cells to the site of injury (Li et al., 2009). In Notch knockout mouse models, perturbation in neovascularization is apparent, which shows that Notch1 binding to its ligand Jagged is involved in the recruitment of stem cells (Kwon et al., 2008). There was reduced CXCR4 expression following Notch knockout (Li et al., 2011b). MCP1 and its receptor CCR2 act as chemotactic signal responsible for homing and differentiation at the site of ischemia (Belema-Bedada et al., 2008). miR-16 family miRNA play a role in G0 to G1 transition (Linsley et al., 2007). Epigenetic control of quiescence can be maintained by activation loci marked by permissible histone marks. For example, H3K4me3-rich genes are functionally important for activation and proliferation (Fuda et al., 2009).

Another likely mechanism could be reprogramming of stromal cells by senescence-associated secretory profile (SASP) produced from injury-induced decidual cells. Animal studies have shown that insult or injury to the endometrium induces decidualization. For example, in mice, intraluminal needle scratch, running a thread through the uterine lumen, or luminal infusion of oil can induce a decidual phenotype. (Deb et al., 2006) (Bany, 2013).

My experimental results show that there is an increase in clonal population following tissue trauma in the miscarriage group, from which we can speculate that there is an activation of the stem cell niche in response to the injury. The endometrial injury is thought to correct asynchrony through the wound healing process (Almog et al., 2010).

Our recent lab finding reveals that decidualization represents a transitional pathway which polarises HESCs into an acutely senescent subpopulation and expanded eMSCs population. The acute senescence phenotype was characterised by an increase in expression of tumour suppressor proteins such as p53, p16, increase in expression of senescence-associated heterochromatin foci proteins such as Histone H1, Macro-H2A and H3K9me3. Nuclear size enlarges and is associated with decreased Lamin B1 expression. This senescent phenotype results in SASP, which drives the decidual inflammatory response. Meanwhile, the expanded eMSCs drives anti-inflammatory responses to decidual cues. This transformation renders the endometrial stroma into the mature decidual matrix, suitable for pregnancy.

Tissue injury is associated with accumulation of senescent cells which enhances the *in vivo* reprogramming of the adult cells. Interleukin 6 (IL-6) a component of SASP, is shown to be essential for the reprogramming process. In mice, the expression of the four transcription pluripotent factors Octamer-binding transcription factor 4, SRY (sex determining region Y)-box 2, Kruppel-like factor 4, C-Myc(OCT4, SOX2, KLF4 and MYC), also induces cellular senescence, and these senescent cells, in turn, produce signals that facilitate the reprogramming of neighbouring cells (Mosteiro et al., 2016).

Emerging evidence has implicated endometrial pro-inflammatory cytokines and increased the abundance of macrophages and dendritic cells (Gnainsky et al., 2015). This is a plausible explanation as a transient inflammatory stromal response upon

decidualization has been shown to induce the expression of receptivity markers (Salker et al., 2012a). However, recurrent miscarriage is linked to an already prolonged and disordered pro-inflammatory decidual response (Salker et al., 2012a, Lucas et al., 2016b). Hence, there is a significant need for a clinical trial to assess the impact of this procedure in recurrent miscarriage patients. Whether the patient-specific response to tissue injury relates to intrinsic differences in clonal HESC populations warrants further investigations.

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Chapter 4

4 Analysis of the link between aberrant decidualization and reproductive failure

4.1 Introduction

Implantation is the rate-limiting step in assisted reproduction treatment (Tan et al., 2014). Clinically, failed implantation is defined by the lack of detectable human chorionic gonadotropin (hCG) levels in either serum or urine 14 days after the intrauterine transfer of one or more embryos. By default, failed implantation is a retrospective diagnosis, rendering it difficult, if not impossible, to discern if caused by a hostile endometrium, impaired embryo quality, or an iatrogenic event. Nevertheless, genome-wide expression studies have identified endometrial signature genes that are predictive of recurrent implantation failure (Tan et al., 2014), defined as the absence of pregnancy following serial transfers of high-quality embryos (Koler et al., 2009, Koot et al., 2016). Interestingly, these studies have also challenged the prevailing concept that implantation failure merely reflects a lack of expression of receptivity genes during the window of implantation (Diaz-Gimeno et al., 2011). Instead, the gene signatures associated with RIF point towards more fundamental defects in cell cycle regulation, cell motility, epithelial-mesenchymal transition (EMT), and signalling pathways involved in stem cell maintenance (e.g. WNT and Notch) (Koot et al., 2016, Koler et al., 2009).

Endometrial stromal cells (EnSCs) play a critical role in the implantation process, not only by relaying hormonal signals to the overlying surface epithelium (Chen et al., 2013, Cooke et al., 1997, Li et al., 2011a), but also by controlling the influx and function of various immune cells, including uterine natural killer cells (Collins et al., 2009, Gellersen and Brosens, 2014a, Nancy et al., 2012). Upon invasion, the implanting embryo is rapidly surrounded and encapsulated by migrating decidualizing stromal cells. As the differentiation process unfolds, gap and tight junctions form between decidualizing cells. Consequently, the conceptus becomes anchored in the

endometrium and forms a matrix that enables coordinated trophoblast invasion (Gellersen and Brosens, 2014a, Wang et al., 2004, Weimar et al., 2013). Growing evidence indicates that decidual cells are exquisitely responsive to embryonic signals and play a critical role in embryo biosensoring and selection (Brosens et al., 2014c, Macklon and Brosens, 2014a, Teklenburg et al., 2010b). On the other hand, decidualization transforms EnSCs into secretory cells that determine the embryonic microenvironment upon breaching of the luminal epithelium. Whether failed implantation can be attributed to an altered endometrial microenvironment that impacts directly on embryo development and survival is not known.

Decidualization of the human endometrium is not dependent on embryo implantation but initiated during the mid-luteal phase of each cycle in response to the postovulatory rise in progesterone and increasing endometrial cAMP levels. Consequently, decidualization is a reiterative process, linked to cyclic activation of mesenchymal stem-like cells (MSCs) and subsequent differentiation into mature stromal cells in regenerating endometrium (Lucas et al., 2016b, Gellersen and Brosens, 2014a). The endometrium harbours abundant MSCs that are multipotent, immuno-privileged, and highly regenerative (Cervello et al., 2011, Gargett et al., 2012a). Functional repair of the endometrium requires activation of poised progenitor cells, induction of transit-amplifying cells and differentiation of mature progeny, which collectively determine the tissue response to deciduogenic and embryonic cues. The mechanisms that control this regenerative pathway and ensure homeostatic balancing of the different stromal cell populations are poorly understood. Arguably, disruption of this process will give rise to dysfunctional EnSCs. Studies on primary EnSC cultures have provided ample evidence that programming defects are linked to reproductive failure. For example, endometriosis is associated with progesterone resistance, defined by the refractoriness of cultured EnSCs to deciduogenic cues (Aghajanova, 2010; Al-Sabbagh, 2012; Klemmt, 2006; Sherwin, 2010; Velarde, 2009; Aghajanova, 2010;

Aghajanova, 2010). By contrast, purified EnSCs from women with a history of recurrent miscarriage mount a prolonged and highly disordered pro-inflammatory response upon treatment with deciduogenic stimuli (Macklon and Brosens, 2014a, Salker et al., 2011b, Salker et al., 2012b, Lucas et al., 2016b).

In this study, we examined the clonogenicity, decidual response and secretome changes in cultured EnSCs purified from mid-luteal biopsies obtained in the cycle prior to Assisted reproductive technology (ART). We demonstrate that successful implantation is associated with coordinated temporal changes in the secretome of differentiating EnSCs that transform a hostile maternal microenvironment into an optimal milieu for implantation. We further show that a disordered EnSC secretome response precedes sporadic failed implantation following ART.

4.2 Results

4.2.1 Successful versus failed implantation: demographic and treatment variables

Twenty women were enrolled in this study; 18 of Caucasian origin, 1 Indian and 1 mixed race. None of the women were smokers. Following IVF, ICSI (n = 10) or frozen embryo transfer (n = 1) in a natural cycle, 10 subjects had a positive pregnancy test, indicating that embryo implantation had occurred. Nine women, including two twin pregnancies, had live births. One subject suffered a pregnancy loss at 6 weeks' gestation. The failed implantation group consisted of 10 subjects. As shown in Table 4.1, there were no significant differences ($P > 0.05$) in patient characteristics, reproductive history, ovarian reserve, treatment characteristics or embryo number and quality, as assessed by standard morphological criteria, between the pregnant and non-pregnant groups. The median time between the biopsy and embryo transfer in the pregnant group was 33 days (range: 30 -36 days) and 35 days (range: 32 - 40) days in the non-pregnant group ($P > 0.05$).

Table 4.1. Demographic and treatment details of the study population

		Pregnant (n = 10)*	Non-pregnant (n = 10)*
Age, years (± SD)		34.0 (±2.9)	35.5 (±3.3)
Duration of infertility, months (± SD)		35.4 (±15.4)	39.0 (±12.9)
Cause of infertility	Male Factor	3	5
	Anovulation	1	2
	Unexplained	3	2
	Mixed	3	1
Previous live births (range)		0.3 (0-2)	0.1 (0-1)
ART cycle number (range)		1.5 (1-3)	1.4 (1-4)
BMI (kg/m2)		24.9 (±3.0)	24.2 (±2.0)
AMH (pmol/L)		15.2 (±13.3)	24.9 (±22.9)
AFC		22.3 (±8.9)	27.9 (±19.6)
IVF		3 (30%)	6 (60%)
ICSI		6 (60%)	4 (40%)
FER		1 (10%)	0
Dose of gonadotropin (IU)		2700.0 (±1002.7)	2448.8 (±872.5)
Duration of stimulation (days)		11.7 (±1.7)	11.5 (±1.3)
Number of oocytes collected		14.6 (±6.4)	14.9 (±8.9)
Single embryo transfer (n)		8	6
Embryos of good quality (%/total)		91.7% (11)	57.1% (8)

*There was no significant difference ($P > 0.05$) in patient or treatment characteristics. BMI: body mass index, AMH: antimullerian hormone, AFC: antral follicle count, ICSI: intracytoplasmic sperm injection, FER: frozen embryo replacement

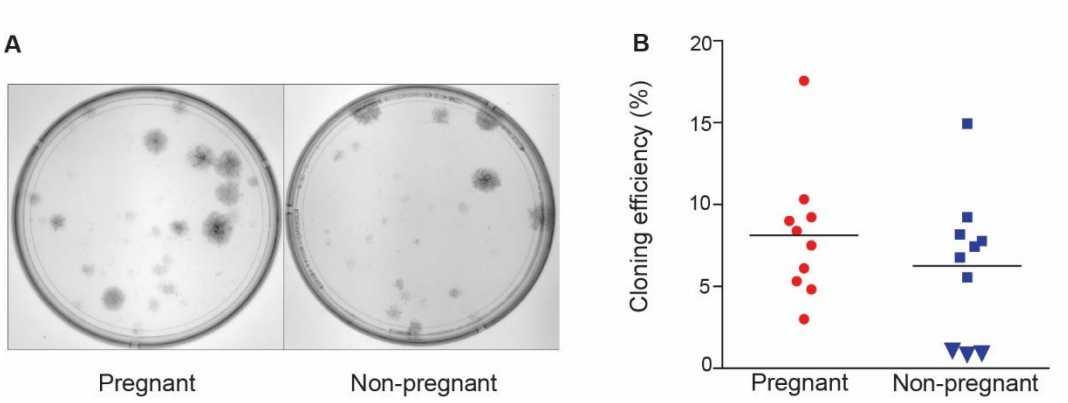


Figure 4.1. The abundance of clonogenic cells in primary EnSC cultures before successful (pregnant) or failed embryo implantation (non-pregnant).

(*A*) representative images of clones obtained after subjecting primary EnSCs to colony-forming assays. (*B*) Colonies with > 50 cells were counted, and cloning efficiency (CE) calculated. Triangles in the failed implantation group indicate primary EnSC cultures deficient in clonogenic eMSCs, defined by CE of ≤ 1%.

4.2.2 Colony-forming unit-fibroblast (CFU-F) activity

To assess the CFU-F activity of EnSCs, frozen endometrial cell suspensions were thawed, expanded in T25 flasks following differential plating, and subjected to colony-forming assays at passage 1 (Figure 4.1). The average endometrial CFU-F activity (mean ± SEM) was 8.1 ± 1.3% and 6.3 ± 1.4% in the pregnant and non-pregnant group, respectively ($P > 0.05$). Notably, endometrial CFU-F activity was ≤ 1% in 3 women who suffered failed implantation, indicating relative MSC deficiency (Figure 4.1).

4.2.3 Responsiveness of EnSCs to deciduogenic cues

For differentiation experiments, EnSCs were plated in 6-well plates, grown to confluency, and then treated or not with 8-br-cAMP and MPA for 2 or 8 days. The median time in culture before the decidualization experiments was 22.5 days (range: 14 - 27 days) and 21 days (range: 12 - 40 days) in the pregnant and non-pregnant group, respectively ($P > 0.05$). The expression of three highly sensitive decidual marker genes, PRL, IGFB1 and HSD11B1, was determined at each time-point by RT-qPCR. As shown in Figure 4.2, decidualization elicited multiple-log increases in the expression of all 3 marker genes; but the level of induction varied greatly between cultures in both the pregnant and non-pregnant group. Furthermore, there was no significant difference ($P > 0.05$) in the expression of these decidual marker genes between the two clinical groups at any of the time-points. Also, the induction of decidual marker genes was not distinct in the 3 cultures exhibiting relative MSC deficiency (Figure 4.2).

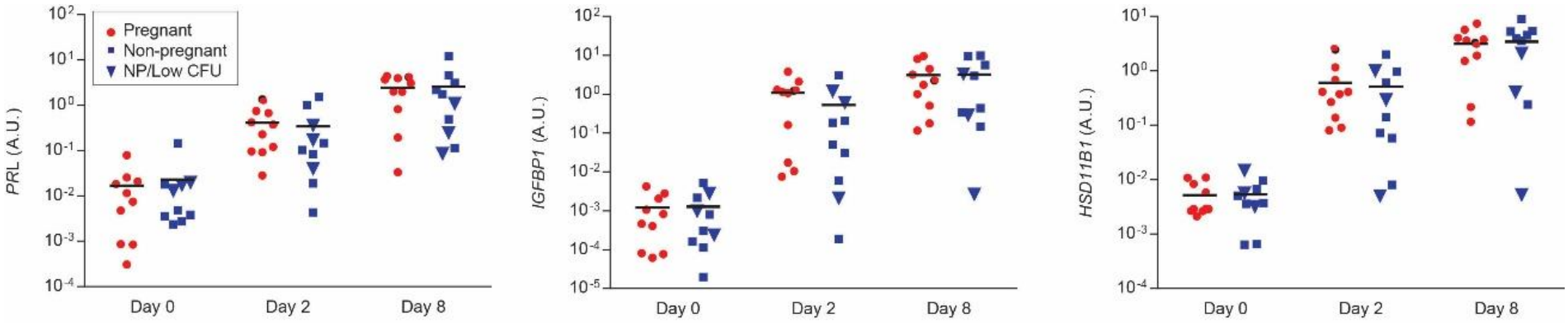


Figure 4.2. Induction of decidual marker genes.

EnSCs from the pregnant and non-pregnant group (n = 10 in each group) were subjected to decidualization for the indicated time-points. Total RNA was then extracted and subjected to RT-qPCR analysis. *PRL*, *IGFBP1* and *HSD11B1* transcript levels were normalised against the levels of *L19* and expressed in arbitrary units (A.U.). The induction of decidual marker genes was comparable between the two groups at each time-point ($P > 0.05$). Note the logarithmic Y-axis. NP/Low CFU denotes cultures from the non-pregnant group with MSC deficiency.

4.2.4 Aberrant EnSCs secretome is associated with subsequent failed implantation

While the expression of many decidual genes, including *PRL*, *IGFB1* and *HSD11B1*, increases exponentially as the differentiation process unfolds, a host of cytokines and immunomodulators are secreted in a biphasic manner, characterized by a rapid rise in response to a deciduogenic signal and followed by a gradual decline (Salker et al., 2012b). We used a multiplex suspension bead immunoassay to measure the secretion of 45 cytokines and immunomodulators in undifferentiated and decidualizing cultures. Out of the 23 factors detectable in a majority of samples, 19 were differentially secreted upon decidualization of cultures for either 2 or 8 days in the pregnant group. The bifurcation in the dendrogram following hierarchical clustering of regulated cytokines demonstrate that the decidual secretome profile is more divergent on day 2 of differentiation when compared to day 8 or day 0 (Figure 4.3). In the pregnant group, differentially secreted factors between undifferentiated cells (day 0) and cells decidualized for 8 days (day 8) included IL-13, CXCL1, IL-6 and IL-8.

The secretome profiles of EnSC cultures from the non-pregnant group were qualitatively different and disordered, illustrated by the lack of bifurcation in the dendrogram (Figure 4.4). Hierarchical clustering of regulated cytokines showed a disordered pattern with both undifferentiated (day 0) and day 8 secretome profiles aligning with day 2 samples. When compared to cultures from the pregnant group, six factors (LIF, IL-6, VEGF-A, VEGF-D, BDNF, and CXCL12) were no longer differentially secreted in cultures associated with ART failure.

Next, we used partial least squares regression (PLSR) analysis to evaluate the temporal multivariate differences in the secretome profiles associated with successful or failed implantation. As shown in Figure 4.5, the secretome profiles were less divergent in cultures from the pregnant compared to the non-pregnant group, at least in undifferentiated cells and cells decidualized for 8 days. The PLSR model indicated

that the inherent secretome differences associated with ART failure were most pronounced in undifferentiated EnSCs. Decidual transformation of the cultures led to a partial convergence in secretome profiles between both clinical groups. Notably, the three most divergent cultures on both day 0 and day 8 in the failed implantation group corresponded to the cultures with the lowest CFU-F activities (Figure 4.1).

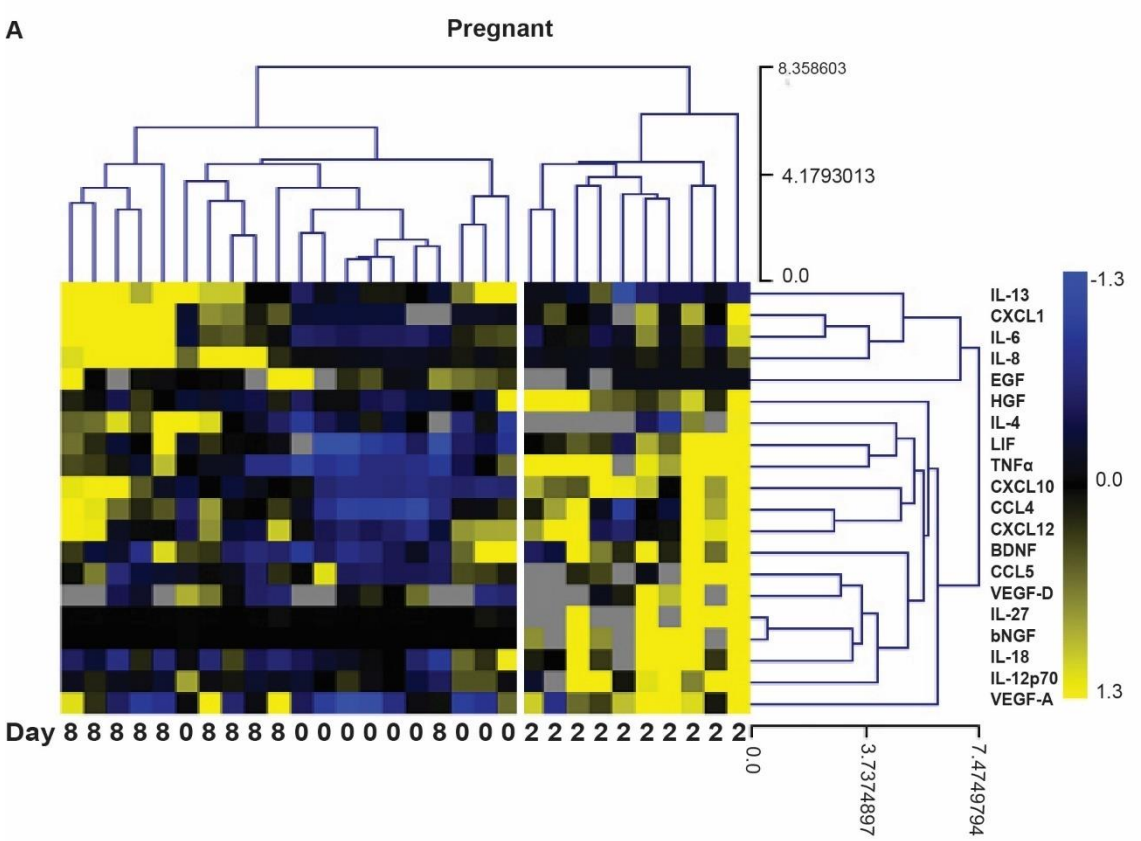


Figure 4.3. Divergent EnSC secretome prior to successful implantation

Differentially secreted factors upon decidualization were hierarchical clustered using a Euclidean distance for the pregnant group. The bifurcation in the dendrogram above the heat map in the pregnant group indicates that the secretory response in decidualizing EnSCs is most distinct after 2 days of differentiation.

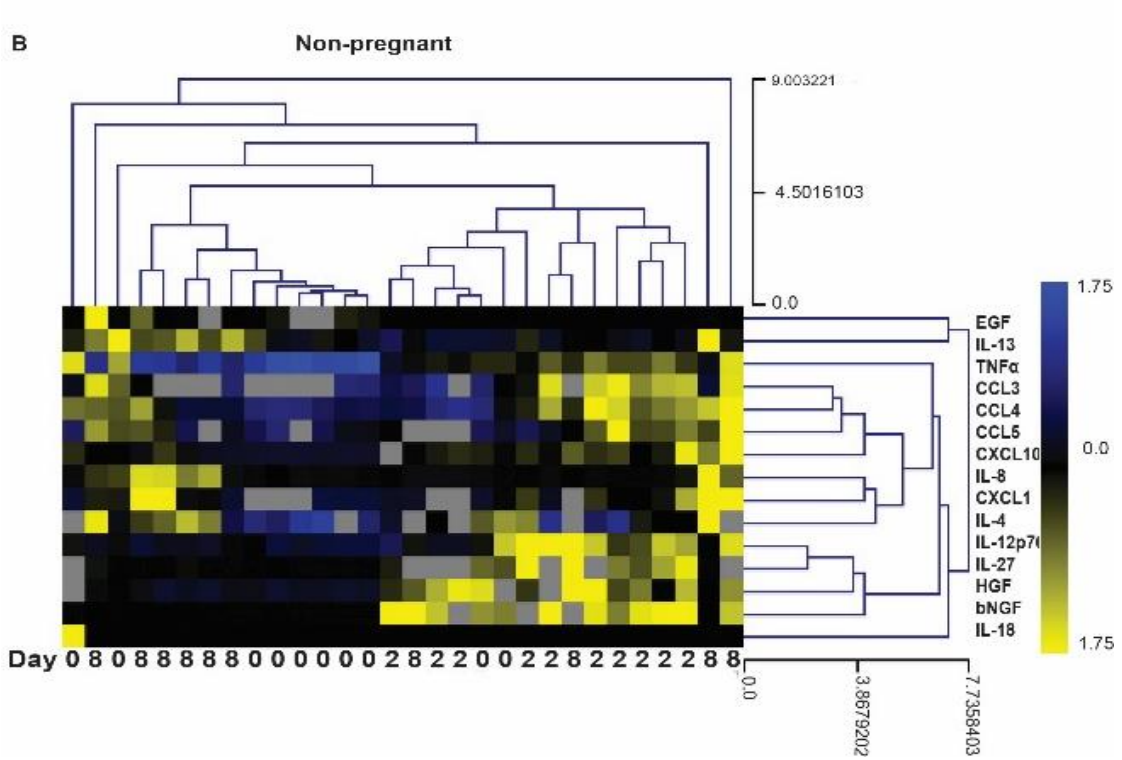


Figure 4.4. Divergent EnSC secretome prior to failed implantation.

The same analysis was performed on decidualizing cultures from the non-pregnant group. By contrast, the temporal secretome changes in cultures associated with implantation failure (non-pregnant) were both qualitatively different and disordered. The colour keys are depicted on the right of the heat maps.

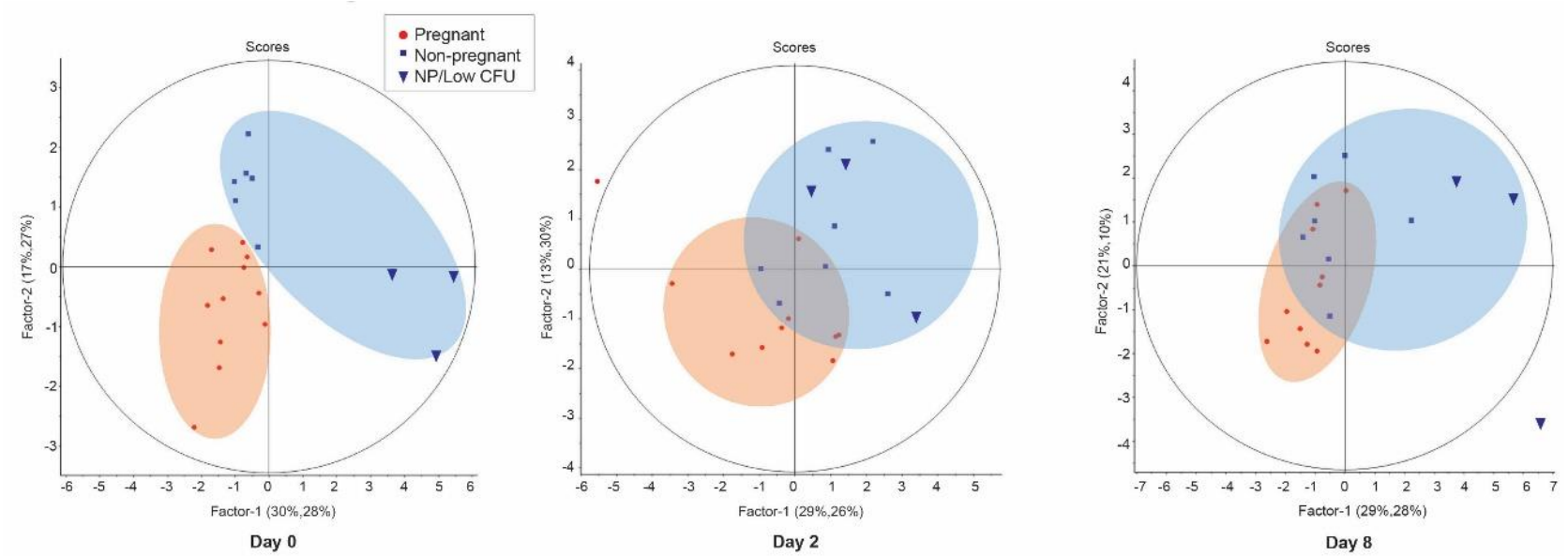


Figure 4.5. Two-dimensional Partial Least Squares (PLS) loading plots of undifferentiated and decidualized secretomes in primary EnSC cultures from pregnant and non-pregnant patients.

PLS regression analysis was used to evaluate the temporal multivariate differences in cytokine and immune-modulatory secretome profile as a result of implantation. The analysis showed that the secretome profiles are less divergent in implantation-positive cultures, at least in undifferentiated cells and cells decidualized for 8 days. Furthermore, the differences in secretomes were most pronounced in undifferentiated cells. Decidualization leads to convergence of secretomes, although not in MSC-deficient cultures (blue triangles). The X-axis represents Principle Component 1 and Y-axis Principle Component 2. NP/Low CFU refers to cultures from the non-pregnant group with MSC deficiency.

Regression coefficient values were used to identify factors that are most closely associated with successful implantation. CCL3, also known as macrophage inflammatory protein 1α (MIP1α), exhibited the highest regression coefficient value in undifferentiated cultures, indicating a positive association with implantation (Supplementary Figure 1). Congruent with the PLSR plots (Figure 4.5), the strength of this association diminished upon decidual transformation of EnSCs (

Supplementary Figure 2). A combination of weakly associated factors was required to generate an association with implantation and secreted factors in decidualizing cells (

Supplementary Figure **2**). For example, a mixture of a positive factor (IL-6) and several negative factors (CCL11, CCL5, IL-18, EGF and VEGF-D), all with regression coefficient less than a magnitude of 0.2, is associated with a successful implantation in cultures decidualized for 8 days.

4.2.5 Impact of the EnSC secretome on human blastocyst development

To understand the impact on embryo development due to temporal changes of decidualizing EnSC secretome, vitrified day 5 human blastocysts were warmed and scored on morphology (Table 4.2). Late day 5 embryos were subjected to assisted hatching and then cultured for 24 hours in 40 µl microdroplets of culture supernatant of undifferentiated EnSCs and cells first decidualized for either 2 or 8 days. Separate pools of culture supernatants were established from the pregnant and non-pregnant groups. The morphology of the embryos was rescored following 24 hours of incubation, and the development of the embryos was evaluated, and growth rates (0, 1 or 2) determined. Increasing growth rates correspond to better development (Table 4.2). Analysis of the embryo growth rates demonstrated that the secretome of undifferentiated EnSCs compromised blastocyst development, exemplified by a growth score of 0 in 13 out of 23 (57%) embryos. By contrast, only 3 out of 34 (9%) embryos had a growth score of 0 when cultured in the supernatant of decidualizing EnSCs (Chi-square test: $P < 0.005$; Figure 4.6 **A**). There was no statistical difference in the growth rates of human blastocysts cultured in the secretome of EnSC decidualized for either 2 or 8 days. Also, no significant differences were found in the growth rates between embryos cultured in medium conditioned by EnSC cultures from pregnant versus non-pregnant patients, whether decidualized or not (data not shown). Finally, the embryonic response to secreted decidual factors was examined more closely by comparing the cytokine content of human blastocysts cultured in the supernatant of EnSCs decidualized for either 2 or 8 days. Multiplex suspension bead

immunoassay allowed quantification of 20 analytes in microdroplets of 18 embryos cultured in medium conditioned by early decidualizing cells (day 2) and 19 embryos cultured in the supernatant of late decidualizing cells (day 8). Following hierarchical clustering, the dendrogram showed that the cytokine/chemokine profiles of embryos cultured in the supernatant of EnSCs decidualized for 2 days were unrelated to either growth rate or clinical group, suggesting an embryo-specific response (Figure 4.6**B**). By contrast, the secretome profiles of embryos cultured in day 8 supernatant clustered, except one sample, with the clinical groups, indicating the lack of a significant embryonic secretory response (Figure 4.6 **C**).

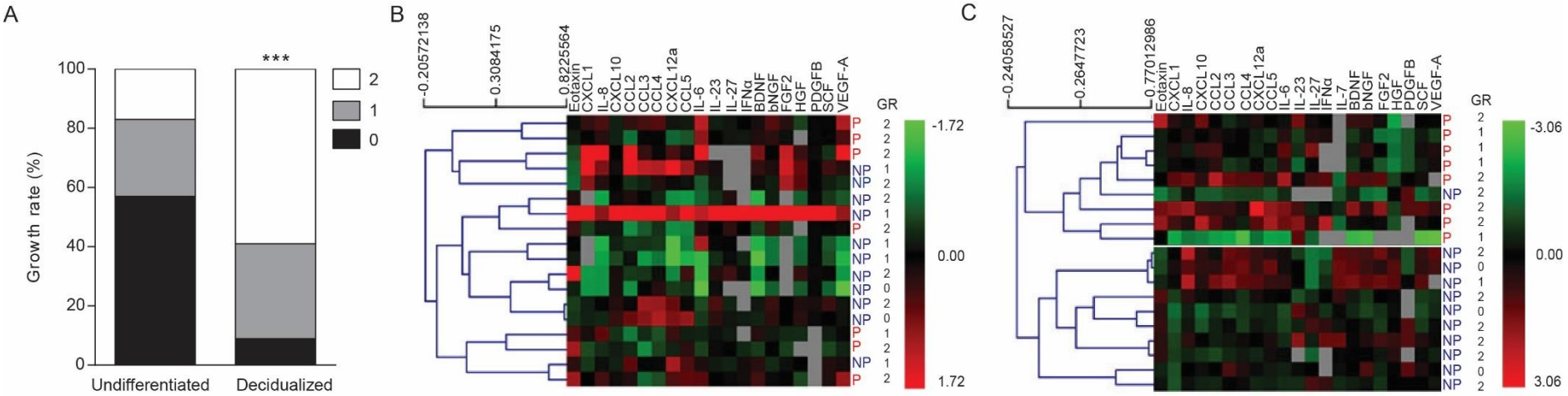


Figure 4.6. The impact of EnSC secretome on human blastocysts.

A, growth rates (scored 0, 1, and 2) of human blastocysts cultured in conditioned medium from undifferentiated (n = 24) and decidualizing EnSCs (day 2 and day 8) (n = 33). *** indicates $P < 0.005$. B, Hierarchical clustering using a Euclidean distance of 20 cytokines and chemokines measured in microdroplets of human blastocysts cultured in the conditioned medium of EnSC decidualized for 2 days. P: pregnant, NP: non-pregnant, GR: growth rate. C, Hierarchical clustering using a Euclidean distance of 20 cytokines measured in microdroplets of human blastocysts cultured in the conditioned medium of EnSC decidualized for 8 days.

4.3 Discussion

In this study, we examined if EnSCs contribute to failed implantation. Study subjects were recruited solely because of the need for ART, irrespective of the cause of infertility or previous treatment cycles. There are various factors that affect egg and embryo quality during an IVF treatment that determines the outcome of the treatment. This might be (i) age and ovarian reserve, (ii) protocol for controlled ovarian stimulation (COH), (iii) sperm quality and (iv) embryo selection. However, the embryo transfer procedure is considered as the rate limiting step in the success of ART.

We reasoned that deficiency of endometrial MSCs could lead to aberrant EnSC function and implantation failure (Lucas, 2016; Barragan, 2016). Although overall CFU-F activity at passage 1 was comparable between the pregnant and non-pregnant group, three cultures associated with failed implantation were deficient in clonogenic MSCs. The secretome of these cultures was markedly divergent from other cultures. This observation supports our hypothesis that the abundance of endometrial stem cell populations is an important determinant of reproductive outcome, although further studies are needed. Overall, the induction of ‘classical’ decidual marker genes was not informative in terms of predicting reproductive success or failure, which was disappointing as the same markers have been widely used to determine the impact of reproductive disorders on the differentiation potential of EnSCs (Klemmt, 2006; Piltonen, 2015; Salker, 2010). However, the level of induction was highly variable between cultures, which probably reflects the clinical heterogeneity of the study population.

For implantation to take place, differentiating EnSCs must transit through distinct functional phenotypes in response to elevated circulating progesterone levels and rising cellular cAMP levels (Gellersen and Brosens, 2014a, Jones et al., 2006b). This transitional pathway is characterised first by an acute auto-inflammatory phase, which

is followed by a profound anti-inflammatory response. The initial auto-inflammatory response associated with the decidual transformation of EnSCs renders the endometrium receptive to embryo implantation (Salker et al., 2012b), whereas acquisition of a mature secretory phenotype enables the endometrium to respond to individual embryos in a manner that either supports further development or facilitates early rejection (Brosens, 2014). Secretome analysis demonstrated that EnSCs from the pregnant group closely phenocopied this transitional pathway in culture, exemplified by the marked and distinct secretory response on day 2 of decidualization. By contrast, this transitional pathway was clearly disordered in cultures from the failed implantation group. In addition, these cultures no longer exhibited temporal changes in the secretion of the main factors involved in inflammation (IL-6), angiogenesis and lymphangiogenesis (VEGF-A, VEGF-D), endometrial receptivity and embryo development (LIF, BDNF) (Cha, 2012; Kawamura, 2009; Kim, 2014; Krussel, 2003; Stewart, 1992).

Notably, the difference in secretome between the two groups of cultures was most pronounced in undifferentiated cells. CCL3, a potent inflammatory chemokine involved in innate immunity as well as wound repair (Chen et al., 2013, DiPietro et al., 1998a), was most strongly associated with successful pregnancy, which emphasises further the importance of transient endometrial inflammation for implantation. Upon decidual transformation, the secretory profiles in both the pregnant and non-pregnant group tended to converge, albeit with some notable exceptions. This is a potentially important observation for some reasons. First, it suggests that endometrial defects associated with reproductive failure could be more prominent in the proliferative phase of the cycle. Second, the convergence in secretomes, if recapitulated *in vivo*, suggests that delaying transfer by a few days could potentially be beneficial for women at risk of implantation failure. Finally, previous studies reported that the

decidual response in cultured EnSCs from recurrent miscarriage patients becomes increasingly divergent as the differentiation process unfolds (Salker, 2010; Salker, 2011; Salker, 2012), which indicates that different pathways are involved in failed implantation and early pregnancy loss.

From the analysis of secretome, we showed that CCL3 in the undifferentiated culture secretome has a positive association with implantation. In response to bacterial infections in the liver, CCL3 expression increases resulting in recruitment of NK cells (Dalod et al., 2002). CCL3 is involved in the recruitment of macrophages to the site of injury, thus improving the angiogenic and collagen production activity, helping in wound repair (DiPietro et al., 1998b). CCL3 was undetectable in endometrial stromal cells, following hormone withdrawal following 12 days of decidualization (Evans and Salamonsen, 2012).

IL-6, as found in the secretome of 8-day decidualized stromal cells, shows a positive correlation with successful implantation. IL-6 is a proinflammatory cytokine and acts on target cells via activation of the JAK-STAT pathway and is involved in stimulation and invasion of trophoblast (Jovanović and Vićovac, 2009, Suman and Gupta, 2014). IL-6 level increases during decidualization and implantation (Godbole and Modi, 2010) and knockout of IL-6 results in endometrial defects and reduced implantation sites (Robertson et al., 2000). It is thought that IL-6 is critical for implantation due to its role in recruitment of immune cells, which is required for trophoblast invasion. Additionally, induced pluripotent cellular reprogramming is associated with an increase in IL-6 expression. SASP associated IL-6 is reported to be critical for inducing pluripotency in the surrounding cells (Chiche et al., 2016).

My work shows a positive correlation of CCL3 and IL-6 with successful implantation. CCL3 might be involved in transient endometrial inflammation during decidualization

thus recruiting immune cells. Likewise, IL-6 induced reprogramming might be vital for the preparing the endometrial environment for implantation of a healthy embryo. Further investigations need to be carried out to affirm this association.

A delicate balance between endometrial receptiveness and selectiveness are required for the successful reproductive outcome. High receptivity with low selectivity is thought to lead to 'super fertility' resulting in recurrent pregnancy loss. Likewise, high selectivity with low receptivity may result in recurrent implantation failure. (Lucas et al., 2016a, Macklon and Brosens, 2014b)

Aberrant decidual responses in purified primary EnSCs culture have been linked to a variety of reproductive disorders, including endometriosis (Aghajanova, 2010; Klemmt, 2006; Velarde, 2009), polycystic ovarian syndrome (Piltonen, 2015), and recurrent miscarriage (Salker, 2010; Salker, 2011; Salker, 2012). These pioneering studies demonstrated that the responsiveness of EnSCs to steroid signals and other cues is not predetermined, but occurs in a disease-specific manner.

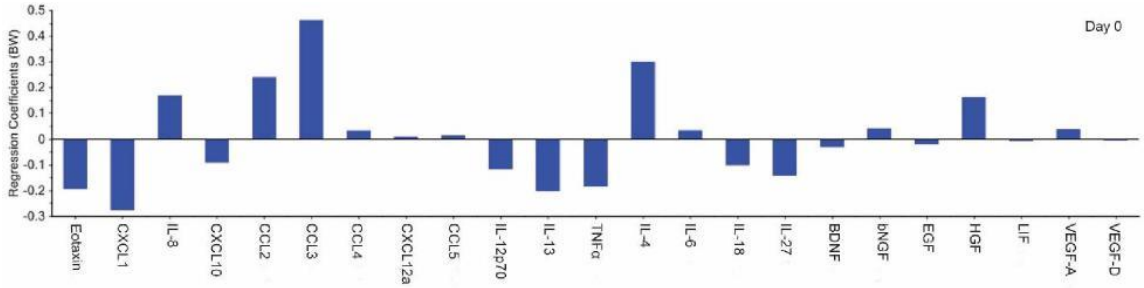
The events that cause implantation failure is unclear. Based on mouse studies, it is widely assumed that unsuccessful implantation reflects the failure of the embryo to breach the luminal epithelium, either because of intrinsic defects in the conceptus or due to a lack of expression of receptivity genes in surface epithelium. Consequently, embryos are thought to remain free-floating in the uterine lumen until they degenerate and die. In humans, this paradigm has been challenged for a number of reasons (Lucas et al., 2013), foremost because human embryos are intrinsically invasive and cannot enter diapause (dormancy) like their murine counterparts. Arguably, the heightened invasiveness may relate to the fact that most human pre-implantation embryos are chromosomally mosaic (Lucas et al., 2016a). Based on genome-wide screening of individual blastomeres, in excess of 70% of high-quality cleavage-stage

IVF embryos reportedly harbor cells with complex large-scale structural chromosomal imbalances, some caused by meiotic aneuploidies but most by mitotic non-disjunction (Fragouli et al., 2013, Mertzanidou et al., 2013, Vanneste et al., 2009). The incidence of aneuploidy in human embryos is estimated to be an order of magnitude higher than in other mammalian species (Fragouli et al., 2013). Differentiated stromal cells are involved in biosensoring of the implanting embryo (Mansouri-Attia et al., 2009). Hence, the luminal epithelium in the human endometrium may not be as important a barrier as in mice. If so, the stromal microenvironment before the window of implantation may be directly embryotoxic, irrespective of the developmental potential of the conceptus.

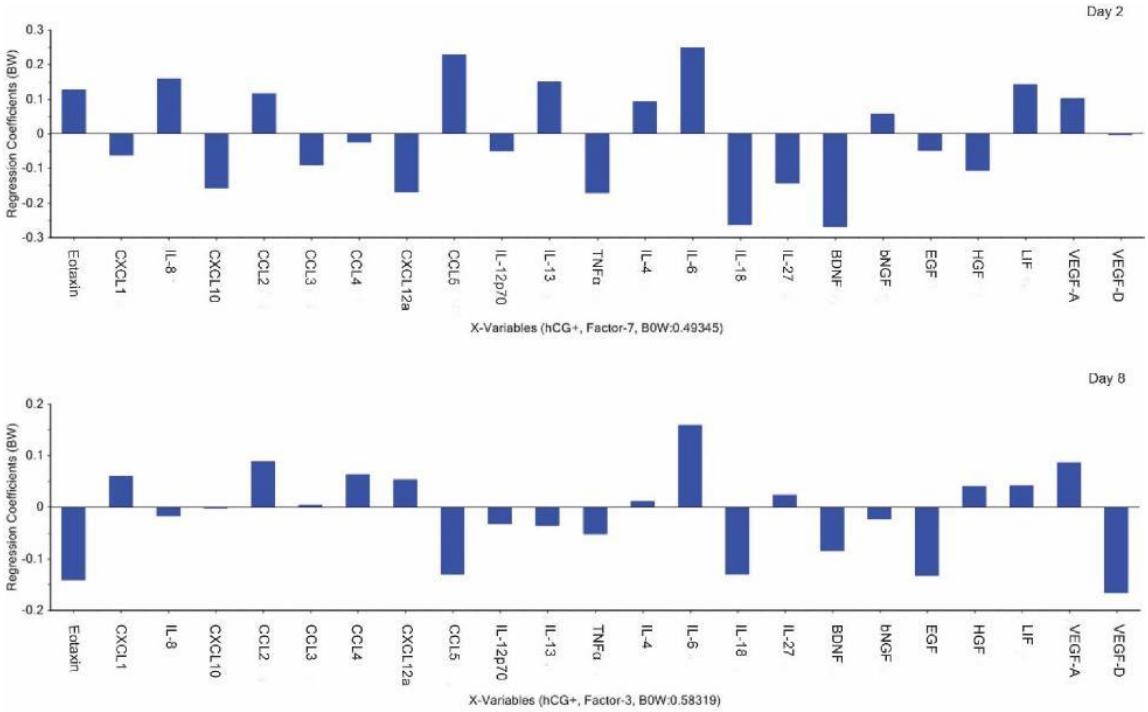
In this chapter, we found that disordered temporal changes in the secretome of decidualizing cultures are associated with subsequent implantation failure. It is evident that a community of resident cells, ranging from immature progenitor cells to mature and senescent cells, makes up the stromal compartment of the endometrium. It is conceivable that the constituents of this community fluctuate from cycle to cycle with transient imbalances contributing to sporadic IVF failure. The effect caused by damaged or deficient progenitor populations is likely to be more severe and could account for recurrent implantation failure.

Analysis of human blastocysts shows that the stromal microenvironment is a major determinant of embryo survival. Our findings demonstrate that the secretome changes associated with undifferentiated EnSCs transitioning to fully decidualized cells induce a graded embryo response, ranging from embryonic growth arrest to a transient embryo-specific secretory response and finally quiescence. Although the EnSC secretome was divergent between the pregnant and non-pregnant groups, no discernible difference in embryo response was observed. This may reflect that EnSC conditioned medium was pooled for the embryo experiments, that only a limited

number of blastocysts were available for these experiments, or that the duration of incubation was too short. Another potential pitfall is that these experiments were performed ‘out-of-phase’ as there is no evidence that day six human embryos are already embedded in the decidualizing stroma. However, there is histological evidence of a Carnegie stage 5a human embryo (post-fertilization age of 7 to 8 days) surrounded by EnSCs. Thus, while our embryo experiments may indeed be ‘out-of-phase’, it is probably only by 1 or perhaps two days.



Supplementary Figure 1. Regression coefficient analysis of non-decidualizing cultures of pregnant group (day 0)



Supplementary Figure 2. Regression coefficient analysis of decidualizing cultures of pregnant group (day 2 or day 8)

Analysis demonstrated that the strength of the association of CCL3 and successful implantation diminishes upon differentiation of EnSCs. In decidualizing cells, a positive factor (IL-6) and several negative factors (CCL11, CCL5, IL-18, EGF and VEGF-D), all with a regression coefficient of < 0.2, were associated with successful implantation.

Table 4.2. Morphology scores and growth rates of human embryos cultured in EnSC conditioned medium

EnSC	Pregnant	Morphology score post warming	Growth rate after 24h of culture
Undifferentiated	Yes	BL2	0
Undifferentiated	Yes	BL3BA	0
Undifferentiated	Yes	BL3CB	0
Undifferentiated	Yes	BL3CA	1
Undifferentiated	Yes	BL2	1
Undifferentiated	Yes	BL4AA	2
Undifferentiated	Yes	BL3BA	2
Undifferentiated	Yes	BL3BB	0
Undifferentiated	Yes	BL3BC	0
Undifferentiated	Yes	BL4CB	0
Undifferentiated	Yes	Collaps	0
Undifferentiated	No	BL3BA	1
Undifferentiated	No	BL2	0
Undifferentiated	No	BL3AB	2
Undifferentiated	No	BL8	2
Undifferentiated	No	BL3BB	0
Undifferentiated	No	BL2	0
Undifferentiated	No	BL4BA	1
Undifferentiated	No	BL3BB	1
Undifferentiated	No	BL3BB	0
Undifferentiated	No	BL2	0
Undifferentiated	No	BL4CB	0
Undifferentiated	No	BL3CB	1
Decidualized (D2)	Yes	BL3BB	2
Decidualized (D2)	Yes	BL4CB	1
Decidualized (D2)	Yes	BL3CB	2

Decidualized (D2)	Yes	BL4BB	2
Decidualized (D2)	Yes	BL3CB	2
Decidualized (D2)	Yes	BLBCB	2
Decidualized (D2)	No	BL4CB	1
Decidualized (D2)	No	BL4BB	1
Decidualized (D2)	No	BL3AA	1
Decidualized (D2)	No	BL3BB	1
Decidualized (D2)	No	BL4BB	2
Decidualized (D2)	No	BL3BC	0
Decidualized (D2)	No	BL2	2
Decidualized (D2)	No	BL3AA	2
Decidualized (D2)	No	BL3AA	2
Decidualized (D2)	No	BL3BB	1
Decidualized (D8)	Yes	BL3BA	2
Decidualized (D8)	Yes	BL3CB	2
Decidualized (D8)	Yes	BL3BB	1
Decidualized (D8)	Yes	BL3BA	1
Decidualized (D8)	Yes	BL3CC	2
Decidualized (D8)	Yes	BL8	1
Decidualized (D8)	Yes	BL2	1
Decidualized (D8)	No	BL3BB	0
Decidualized (D8)	No	BL3BA	2
Decidualized (D8)	No	BL3AA	2
Decidualized (D8)	No	BL3CB	2
Decidualized (D8)	No	BL3CC	1
Decidualized (D8)	No	BL3BB	2
Decidualized (D8)	No	BL3BB	2
Decidualized (D8)	No	BL4BB	2
Decidualized (D8)	No	BL3AC	0
Decidualized (D8)	No	BL4BB	2
Decidualized (D8)	No	BL4CB	2

Embryos were scored on morphology post warming using the Gardner and Schoolcraft criteria. After culture for 24h in EnSC supernatant, embryos were morphologically evaluated and scored with growth rates 0, 1 or 2 to indicate development based on expansion and morphology scores of ICM and TE: 0: embryo was degraded or did not develop further; 1: embryo moderately expanded or significantly expanded further accompanied with a “C”-score for the TE and/or ICM; 2: embryos significantly expanded with ≥ “B”-score for the TE and/or ICM

Chapter 5

5 In-depth characterization of eMSCs / ePCs cells.

5.1 Introduction

The endometrium is an excellent model of controlled tissue regeneration and remodelling. Its regeneration is unparalleled as the endometrium increases to a thickness of up to 6-8 mm in a week's time following menstruation. This vast regenerative potential of the endometrium is presumed to be attributed to the presence of epithelial and mesenchymal stem/progenitor cells, which has been reported in recent years (Gargett et al., 2007, Gargett et al., 2016, Meng et al., 2007a).

Following each menses, the endometrium undergoes physiological angiogenesis to facilitate regeneration and implantation (Gargett, 2007 239; Barragan, 2016 49). Perturbations in cyclical remodelling or effective communication between the endometrium and embryo may result in pregnancy complications including infertility and implantation failure (Evans et al., 2016, Brosens et al., 2014b).

The dynamic endometrium is comprised of two major zones: functionalis, a transient layer with glands extending from surface epithelium along with supportive stroma and basalis. Basalis layer consists of a basal layer of glands, stroma, vasculature and lymphoid aggregates. Functionalis layer is shed during menstruation and regeneration starts from basalis layer (Padykula et al., 1984, Padykula, 1989, Jabbour et al., 2006). Specific or combination of markers has been reported for perivascular cells capable of regeneration localised around blood vessels in functionalis layer and as well as in a basal layer of the endometrium (Gargett et al., 2016). Sushi domain containing-2 (SUSD2) antigen, was used to isolate perivascular cells using magnetic activated cell sorting (MACS) method with a W5C5 antibody, specific to the antigen (Masuda et al., 2012b). Endometrial stem cells (eMSCs) can be purified on the basis

of their coexpression of two perivascular cell surface markers- CD140β and CD146. This method requires using fluorescence-activated cell sorting (FACS) or two sequential magnetic sorting (Schwab and Gargett, 2007). Sequential magnetic bead selections may also affect the cell viability during cell sorting. The single marker SUSD2 magnetic cell purification is less damaging and resulting in high yield of cells in comparison to FACS isolation (Schwab and Gargett, 2007, Masuda et al., 2012a).

Since there are several terminologies for cellular subpopulation within endometrium, to uncomplicate, we have come up with new nomenclature based on niche and their potency, listed in Table 5.1.

The unique property of human endometrium is its sequential regulation of proliferation, decidualization and shedding of the functionalis layer through every menstrual cycle (Gellersen and Brosens, 2014a).Progesterone induced decidualization commences from the endometrial stromal cells surrounding the spiral arteries. Endometrial stem/progenitor cells (SUSD2^{+}/ePCs, CD146^{+}/PDGFR-β^{+}) are reported to be localised around the spiral arteries throughout the endometrium. A recent study reported that menstrual phase has cells of higher self-renewal capability and more stem/progenitor population could be isolated from the basal layer of the endometrium (Shan et al., 2016). These perivascular cells, when decidualized in vitro, produced higher levels of cytokines and chemokines that are responsible for inducing decidualization and may play a role in homing of trophoblast cells to the spiral arteries (Murakami et al., 2014, Evans et al., 2016).

ePCs account for 7.0 ± 0.5% of the total endometrial stromal cell population and it is enriched with clonal cells compared to EnSCs (Masuda et al., 2012b, Murakami et al., 2013a). Clonogenicity is the ability of the single cell to multiply and produce progeny of its own when seeded at low cellular density. Colony forming unit-fibroblast (CFU-

F) assay is a primary assay to identify the presence of stem cells within any tissue (Teixeira et al., 2008). Human endometrium has clonogenic epithelial (0.15%) and stromal (1.3%) population (Chan et al., 2004). For the culture of the clonogenic population *in vitro,* there is a vital need for growth factors, epidermal growth factor in the case of epithelial cells and basic fibroblast growth factor (bFGF) in the case of clonogenic endometrial stromal cells (Schwab et al., 2005).

ePCs cells exhibit differentiation potential and have the ability to form adipocytes, chondrocytes, osteoblasts (Masuda et al., 2012b) and smooth muscle cells (Su et al., 2014). Cultured EnSCs can differentiate into a single lineage, and the potential is lesser extent compared to eMSCs (Gargett et al., 2016). Studies show that cultured EnSCs differentiated into chondrocytes (Wolff et al., 2007), pancreatic β like cells (Santamaria et al., 2011), neurogenic (Wolff et al., 2011), megakaryocytes (Wang et al., 2012b) and oligodendrocyte precursors (Ebrahimi-Barough et al., 2013).

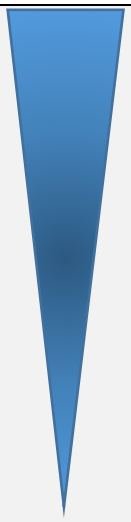
Characterization of ePCs and EnSCs cells revealed that over 12 days of *in vitro* culture EnSCs became positive for the SUSD2 epitope. The SUSD2 expression is contact dependent and is under the control of Notch signalling pathway (Murakami et al., 2014). Gene profiling of cultured ePCs cells shows that these cells have a perivascular, pericytic gene signature suggestive of a role in angiogenesis during regeneration following in the case of placentation and in the absence of implanting embryo, menstruation (Evans et al., 2016).

Pluripotent stem cells that are committed to their lineage are capable of forming organoids, which is defined as a collection of the self-organizing structure. (Lancaster and Knoblich, 2014b). There is a major leap in the field of organoid formation from pluripotent stem cells, (Eiraku and Sasai, 2012, Eiraku et al., 2011, Eiraku et al., 2008, Nakano et al., 2012, Sato et al., 2009, Suga et al., 2011). There have been several

attempts to establish endometrial epithelial organoid cultures (Blauer, 2012, Rinehart Jr et al., 1988). Studies have shown that a 3D layered structure could be created using epithelial and stromal cells, as a model for the attachment of trophoblast cells *in vitro* (Wang et al., 2012a). Endometrial stromal cell and embryo co-culture systems have demonstrated that active embryo selection by decidualized stromal cells is critical for reproductive success (Brosens et al., 2014b). Understanding embryo implantation may help to enhance the success of ART (Koot et al., 2012).

In this chapter, I demonstrate that freshly isolated ePCs and EnSCs cells are divergent not only in their gene expression profile but also functionally. I have characterised mesenchymal stem cells (eMSCs), a subpopulation of ePCs and transit amplifying cells (eTAs) a subpopulation of EnSCs, based on their gene expression pattern, clonal ability under varying oxygen concentration, and their angiogenic potential. I have observed a unique potential for eMSCs to organise themselves into a macroscopic 3D structure when seeded on Matrigel in the presence of angiogenic media, termed ‘Endometrial Regenerative Bodies (ERBs) Further analysis on the ability of ERBs to differentiate, re-epithelialize was carried out.

Table 5.1. Nomenclature for cell types within the endometrium.

Cell Type	Marker	New terminology	Stemness
Clonal SUSD2^{+}	Marker undefined	Endometrial mesenchymal stem cells ((eMSCs)	
Perivascular cells	SUSD2^{+}	Endometrial pericytes ((ePCs)	
Clonal SUSD2^{-}	Marker undefined	Endometrial transit amplifying cells ((eTAs)	
Endometrial stromal cells	SUSD2^{-}	Endometrial stromal cells ((EnSCs))	

5.2 Results

5.2.1 Gene signatures of ePCs and EnSCs

To characterise the constituent perivascular and non-perivascular cell populations of the endometrial stromal compartment, single-cell suspensions were prepared from three mid-luteal biopsies and separated into ePCs and EnSCs cell fractions by magnetic-activated cell sorting (MACS). RNA sequencing profiled the transcriptomes of these samples. Per sample, on average, 22 million single-end reads were sequenced. Z-scores of the transcripts-per-million (tpms) values for the differentially expressed genes were calculated and a heat map of this association is depicted in Figure 5.1A. Based on tpms, out of 23,712 expressed genes, within ePCs, 13 genes were upregulated (Figure 5.1 A), and 232 genes were downregulated. List of downregulated genes with $P < 0.05$ are listed in Appendix 2. With further stringent analysis, it was found that 27 genes were upregulated exclusively in EnSCs (Appendix 3).

Of these 13 genes, SUSD2 was the highest differentially expressed gene between the two groups. (Figure 5.1 B). Gene Ontology (GO) annotation of this gene list showed that these genes were involved in various biological processes including cell adhesion ($P = 0.03$), cell contraction, metabolic processes and response to stimuli.

qRT-PCR on six samples from the same populations used in the RNA-Seq validated differential gene expression between the two groups. (Figure 5.2). To test whether freshly MACS isolated ePCs retain any *in vivo* phenotypic markers, I examined the endometrial tissue distribution of 13 differentially expressed genes using The Human Protein Atlas (http://www.proteinatlas.org/). I successfully annotated 12 out of 13 genes and identified seven genes- *ANGPT2, CCDC102B, DCLK1, EPHA3, GUCY1A2, OLFML2A, POSTN* that share the same restricted tissue distribution as

SUSD2, characterised by prominent immunostaining surrounding the endometrial arterioles (Figure 5.3) (Table 5.2).

Data mining of Gene Expression Omnibus (GEO) database showed the changes in the expression levels of five of the perivascular niche genes- *CNTN1, DCLK1, GUCY1A2, OLFML2A, PDE1A* over the course of a menstrual cycle (

Figure 5.4). These genes were expressed higher in proliferative phase and reduced towards the late secretory phase. This shows that these perivascular niche cells involvement in the proliferation of the endometrium following menstruation.

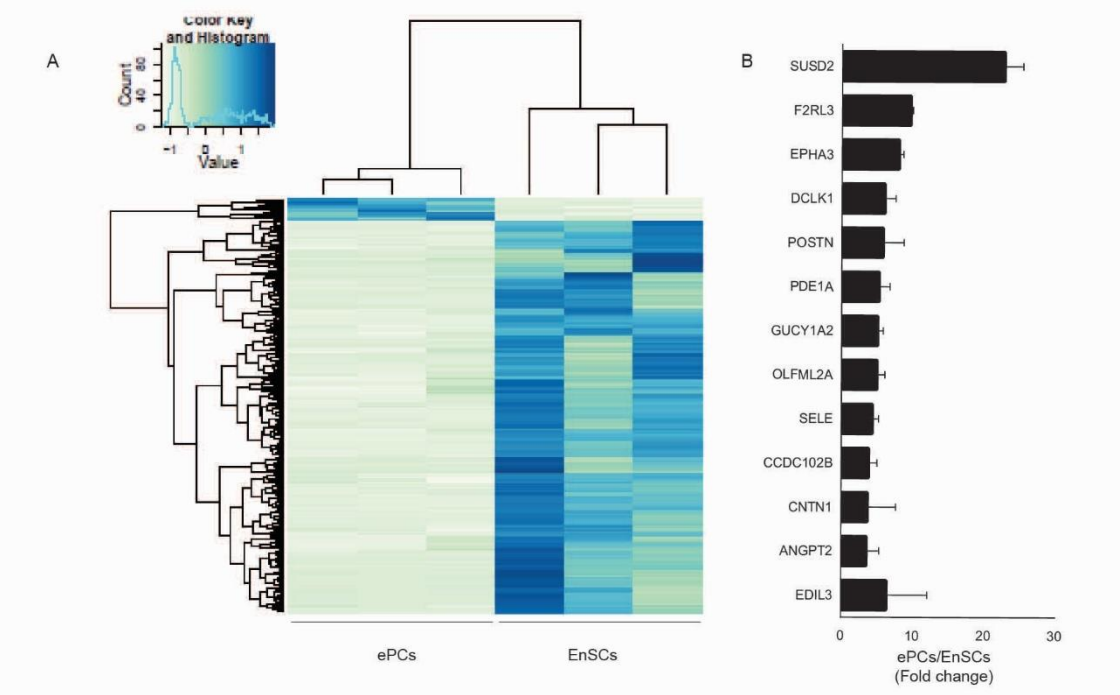


Figure 5.1. Gene signature in ePCs.

A**.** Clustered heat map of top-ranked differentially expressed transcripts in three paired freshly isolated ePCs and EnSCs. B. Pairwise analysis of RNA-sequencing data showing fold enrichment in ePCs cells of novel endometrial perivascular markers. Data represent mean ± SEM.

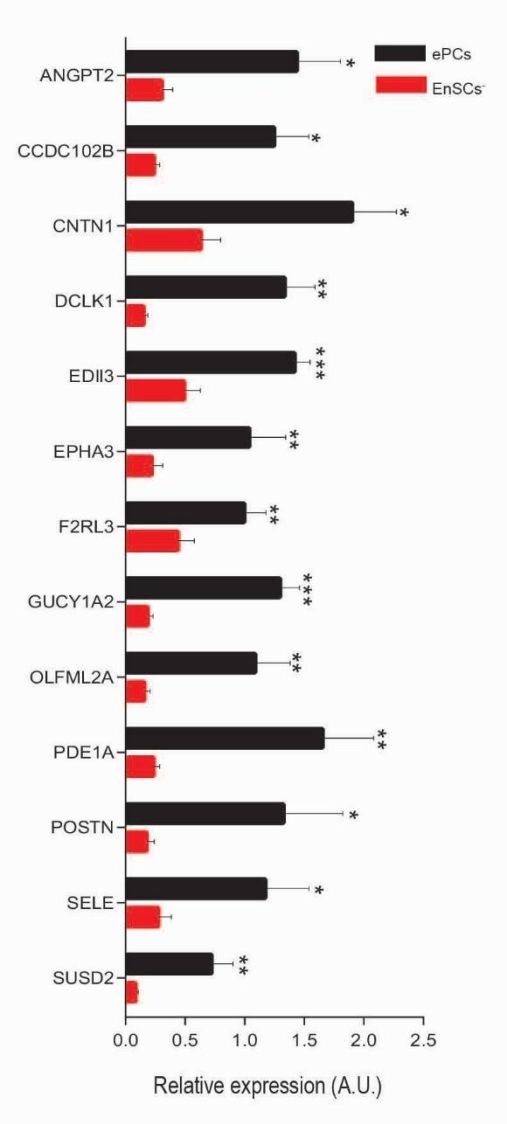


Figure 5.2. Validation of RNA sequencing using RT-qPCR.

mRNA transcript expression of 13 differentially expressed genes in freshly isolated ePCs and EnSCs in six paired biological replicates. mRNA was harvested after separation of cells from fresh biopsies and transcript expression analysed by qRT-PCR. *** indicates $P < 0.001$. ** indicates $P < 0.01$. * indicates $P < 0.05$. Data represent mean ± SEM.

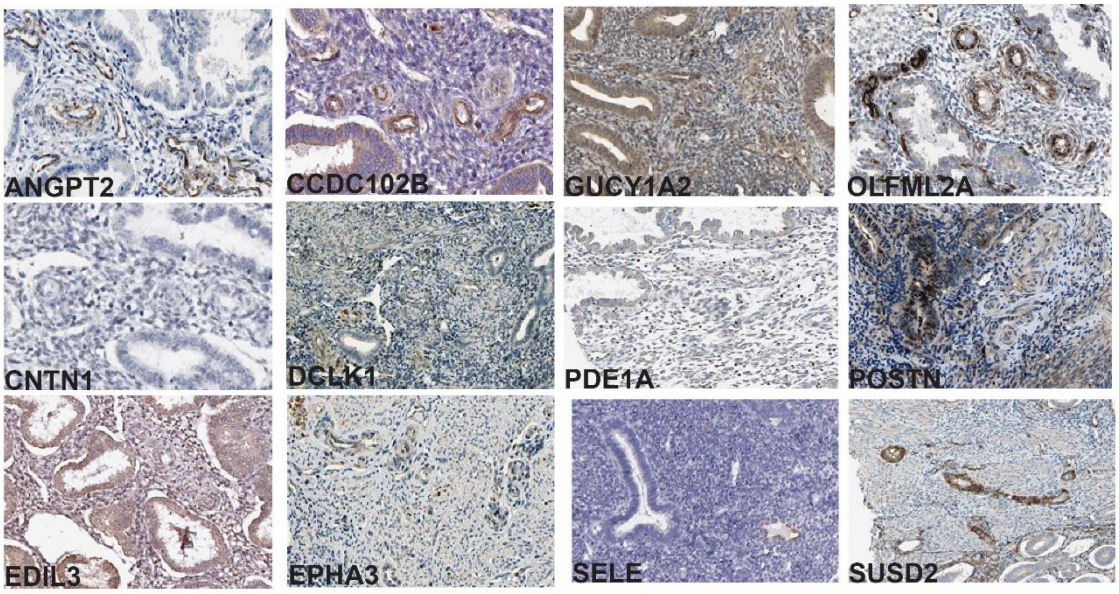


Figure 5.3. Tissue distribution of perivascular genes enriched in freshly MACS isolated ePCs.

The micrographs were obtained from The Human Protein Atlas (http://www.proteinatlas.org/).

Table 5.2. Histologic expression of perivascular niche genes.

Gene Name	Stroma Staining	Glandular Staining	Perivascular Staining
ANGPT2	Low	Not detected	Low
CCDC102B	Low	Medium	Low
CNTN1	Not detected	Low	Not detected
DCLK1	Not detected	Low	Low
EDIL3	Low	Medium	Not detected
EPHA3	Not detected	Low	Low
F2RL3	Not listed	Not listed	Not listed
GUCY1A2	Medium	Medium	Medium
OLFML2A	Not detected	Low	Low
PDE1A	Medium	Medium	Not detected
POSTN	Not detected	Low	Low
SELE	Not detected	Not detected	Not detected
SUSD2	Low	Medium	Medium

Tissue distribution of perivascular niche genes based on the micrographs obtained from The Human Protein Atlas (http://www.proteinatlas.org/).

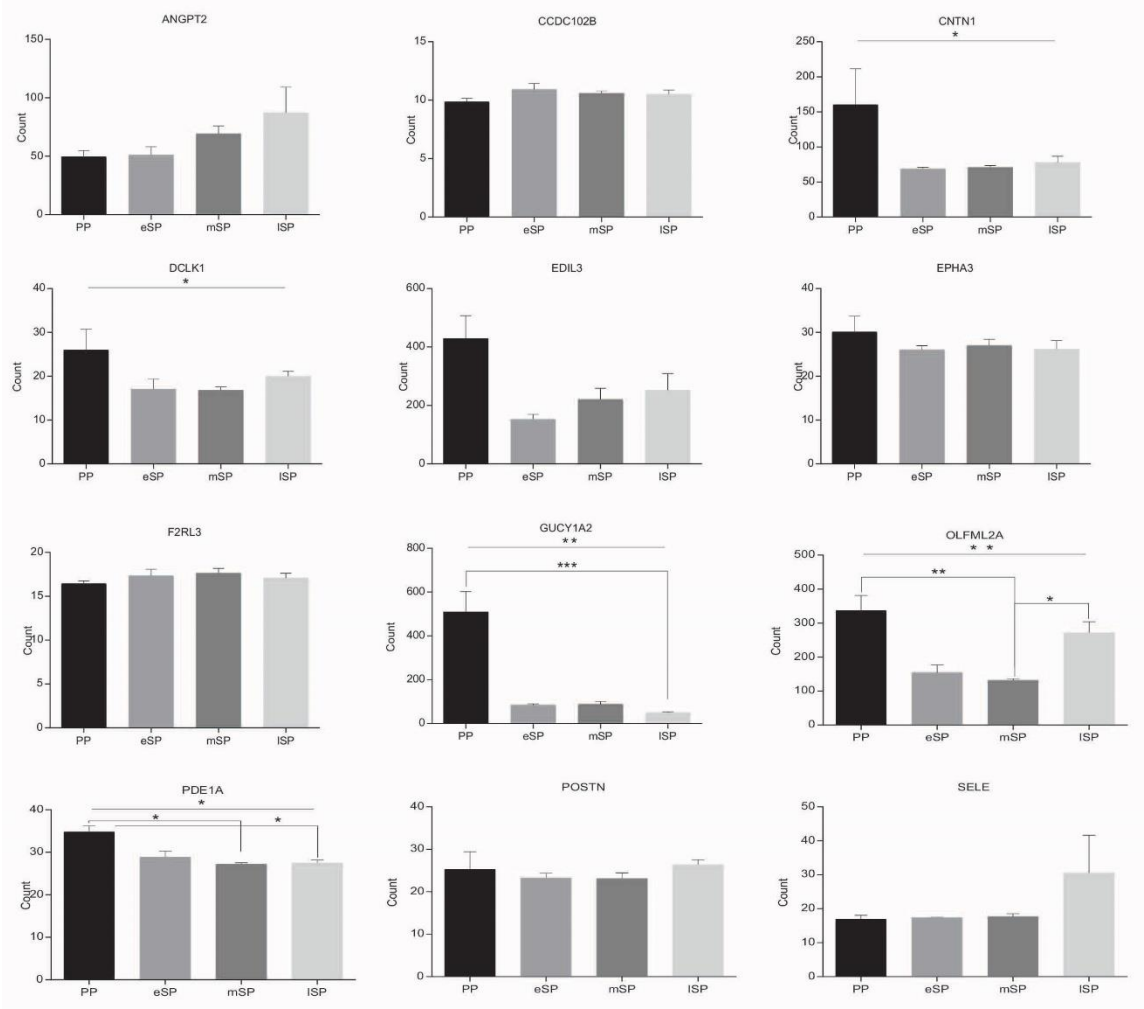


Figure 5.4. Expression of perivascular niche genes through the menstrual cycle

GEO profile microarray of genes expressed by ePCs during the proliferative, early secretory, mid-secretory and late secretory phases of the menstrual cycle in 28 subjects using Affymetrix Human Genome U133 Array. *$P < 0.05$; ** $P < 0.01$; ***$P < 0.001$. Data are presented as means ± S.E.M

5.2.2 Functional characterization of ePCs and EnSCs

Stem cell niche cells maintain the homoeostasis of the tissue by continuous and rapid replacement of differentiated cells. This renewal process occurs by rapid proliferation and subsequent migration (Leedham et al., 2005). Since ePCs and EnSCs are localised around blood vessels, I wanted to further assess their niche properties by characterising their proliferation, migration and contractile ability.

Real-time monitoring of proliferation was carried out using xCELLigence. Freshly isolated ePCs and EnSCs cells from three mid-luteal biopsies were cultured in the xCELLigence proliferation assay plate and proliferation monitored over three days. Proliferative capacity did not vary between ePCs and EnSCs during the first 20 hours of monitoring. However, ePCs cells showed a massive increase in proliferation following this time point. (

Figure **5.5** A). The proliferation of ePCs increased (5-30 fold) over time and reached maximum towards 72 hours whereas EnSCs was slow and maintained a steady level.

Real-time migration was monitored in freshly isolated ePCs and EnSCs populations using the xCELLigence system. Cells were seeded onto a double chambered migration assay plate, with basic fibroblast growth factor (bFGF)

used as a chemoattractant. Analogous to the results obtained from the proliferation assay, the rate of migration was no different between ePCs and EnSCs cells up to 15 hours, after which the rate of migration was higher in ePCs cells (

Figure **5.5** B). After 32 hours, the migration tails off as the cells have migrated covered the surface of the bottom chamber of the migration assay plate.

The contraction ability of ePCs and EnSCs cells was measured using depth sensing indentation device in collaboration with School of Engineering, the University of Warwick, following the previously described protocol (Jin et al., 2016). Cells were mixed with collagen and were allowed to polymerise in 35mm dishes. Following stimulation, the contraction force was measured using the device. The contraction force is significantly higher in ePCs cells (*P* < 0.05) (

Figure **5.5** C).

Taken together, these observations show that proliferation, migration, and contraction are enhanced in ePCs population in the endometrial stroma, showing their perivascular niche origin.

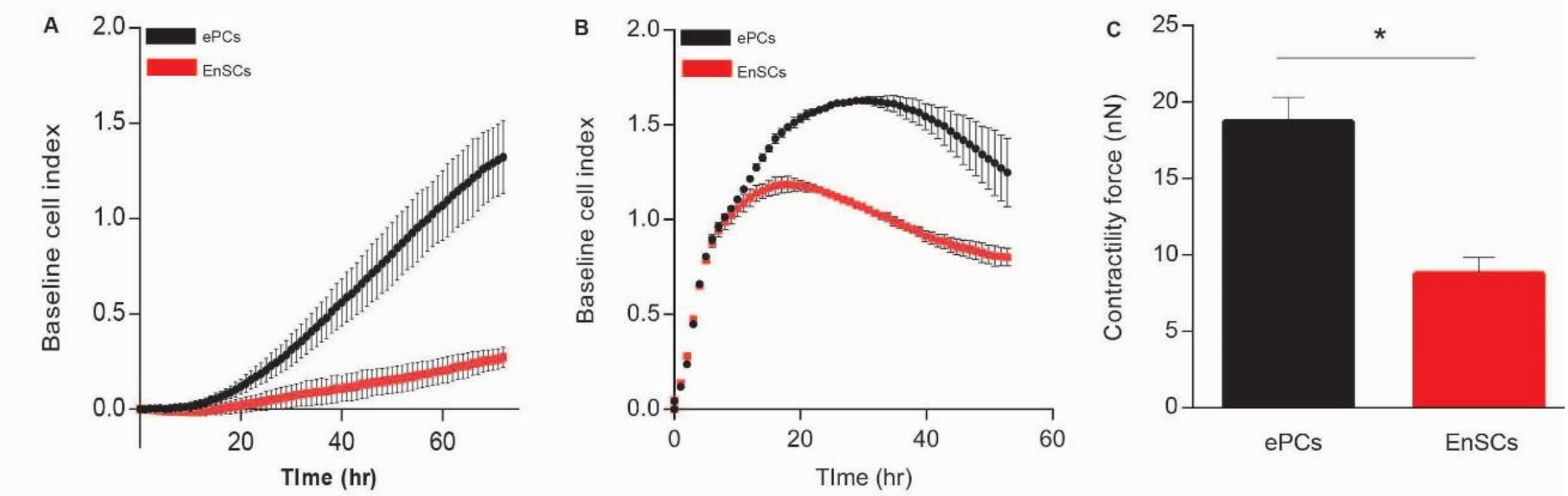


Figure 5.5. Functional characterization of ePCs and EnSCs cells.

A. Real-time monitoring of proliferation of ePCs or EnSCs cells over 60 hours using xCELLigence RTCA system (n=3). B. Real-time monitoring of migration of ePCs or EnSCs cells over 50 hours using xCELLigence RTCA system (n=3). C. Measurement of contractility force between ePCs and EnSCs cells using depth sensing indentation device. All experiments were carried out in three paired biological replicates (n=3). * indicates $P < 0.05$. Data represent mean ± SEM.

5.2.3 Oxygen consumption of ePCs and EnSCs cells

Mitochondrial respiration was measured using Seahorse XF Extracellular Flux Analyzer by injecting mito-stress agents such as Oligomycin, Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) and Rotenone. Oligomycin inhibits ATP synthase (complex V) and the decrease in oxygen consumption rate (OCR) following injection of oligomycin correlates to the mitochondrial respiration associated with cellular ATP production. FCCP is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential. As a result, electron flow through the electron transport chain (ETC) is uninhibited, and oxygen is maximally consumed by complex IV. The FCCP-stimulated OCR can then be used to calculate spare respiratory capacity, defined as the difference between maximal respiration and basal respiration. Spare respiratory capacity is a measure of the ability of the cell to respond to increased energy demand. The third injection is rotenone, a complex I inhibitor. Rotenone shuts down mitochondrial respiration and enables the calculation of nonmitochondrial respiration driven by processes outside the mitochondria (Tan et al., 2015).

There was no significant difference in the basal respiration before injection of Oligomycin. Following the injection of Oligomycin, there was no significant difference in the adenosine triphosphate (ATP) turn over. However, after FCCP injection, the oxygen consumption reached the maximal respiration (Figure 5.6 A), and there was a clear difference between OCR of ePCs and EnSCs cells ($P = 0.02$) (Figure 5.6 B). When rotenone was injected, mitochondrial respiration of both cell fraction was shut down, and there was no difference in non-mitochondrial respiration. Overall there was a significant difference in the oxygen consumption rate between ePCs and EnSCs cells as measured by Seahorse XF Bioanalyzer.

This suggests that ePCs cells are less metabolically active. Furthermore, the extracellular acidification rate (ECAR) was measured high in ePCs cells compared to EnSCs cells. These results indicate oxygen consumption is lower in ePCs cells, indicative of that these are perivascular cells or stem cell niche cells.

5.2.4 Colony forming unit- fibroblast (CFU-F) activity under hyperoxia and physoxia

Since oxygen consumption was lower in ePCs cells, I wanted to analyse whether oxygen concentration has an impact on the stem cell population. To evaluate this, CFU-F were carried out under a normal laboratory oxygen concentration-hyperoxia (21% O_2) and a low oxygen concentration-physoxia: a near physiological oxygen concentration (3% O_2) (Figure 5.7 A). We have chosen 3% oxygen concentration as mesenchymal stem cell niche's oxygen concentration was reported to be 2-8% (Kofoed et al., 1985, Harrison et al., 2002). At 21% O_2, eMSCs formed small sized colonies, whereas no or very few colonies formed in eTAs. At 3% O_2, the number of colonies formed by TAs was significantly higher (Figure 5.7 B) whereas eMSCs formed larger colonies with a greater surface area than at 21% O_2 (Figure 5.7 C). Overall, the low oxygen concentration has a positive impact on the size and number of the colonies derived from both ePCs and EnSCs. Oxygen concentration similar to niche condition favours the growth of colonies.

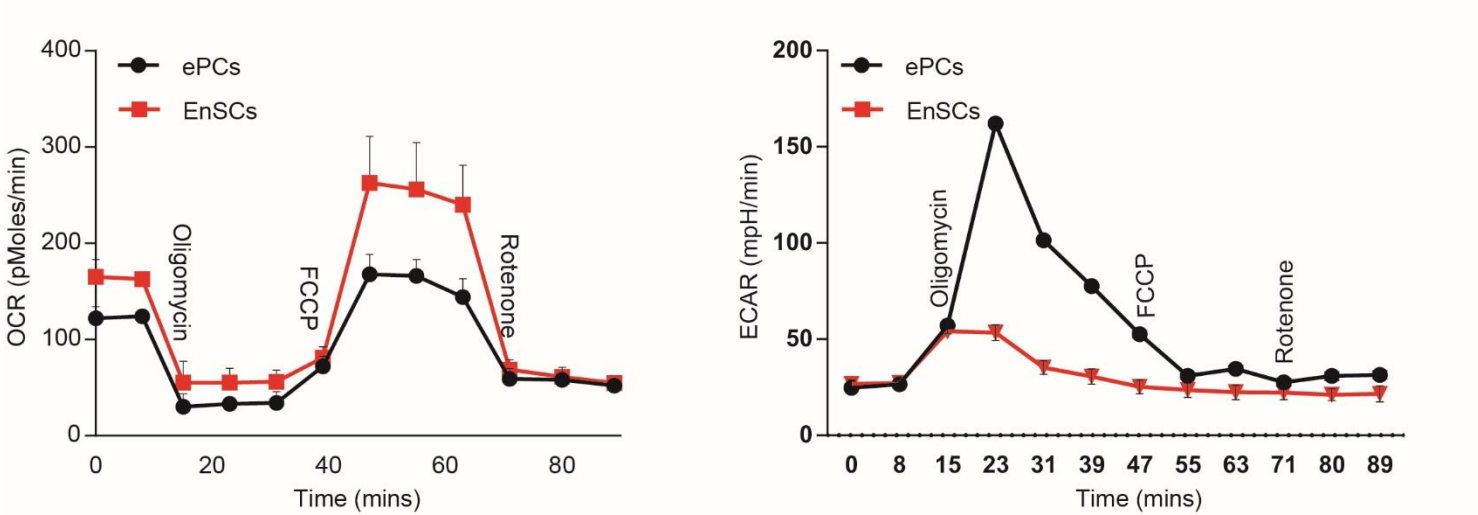


Figure 5.6. The metabolic profile of ePCs and EnSCs.

The difference in metabolism was measured by oxygen consumption rate (OCR) and extra cellular acidification rate (ECAR) using Seahorse XF Analyzer in three paired ePCs and EnSCs. Mito stress compounds were injected sequentially and oxygen consumption rate was measured (n=6). Data represent mean ± SEM.

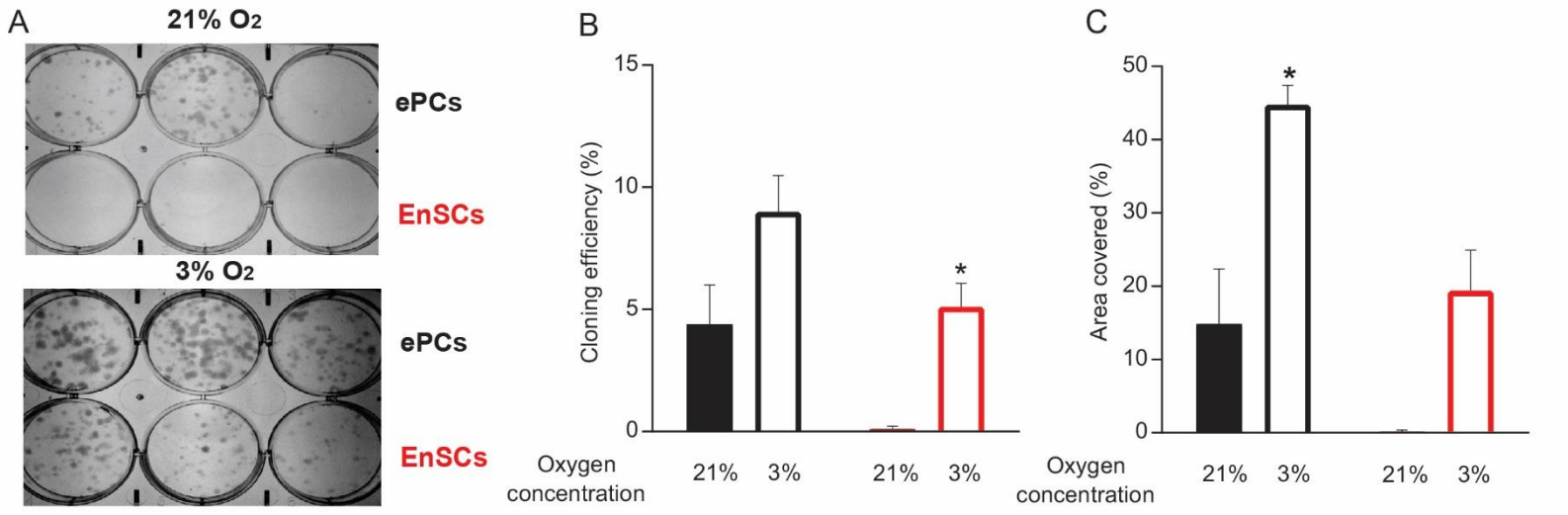


Figure 5.7. Cloning efficiency under 21% O_2 (hyperoxia) and 3% O_2 (physoxia).

A. Representative images of clones obtained after subjecting ePCs/EnSCs to colony forming unit assays (CFU-F) under 21% oxygen and 3% oxygen. B. Colonies with > 50 cells were counted, and cloning efficiency (CE) calculated for three paired ePCs/EnSCs replicates. C. Area of colonies was measured using Adobe Photoshop (n=3). * indicates $P < 0.05$. Data represent mean ± SEM.

5.2.5 O_2 dependent regulation of PV gene signature in eMSCs and eTAs under 21% O_2 and 3% O_2

Next, I wanted to study the expression of perivascular specific gene expression in endometrial eMSCs and eTAs. For this, CFU-F was carried out on MACS separated eMSCs and eTAs with cells at a clonal density of 50 cells/cm^2. eMSCs and eTAs were cultured under 21% O_2 and 3% O_2 for 15 days, RNA was harvested and cDNA was synthesised. RT-qPCR was carried out for reference gene *L19* and 13 genes which were highly expressed in RNA-seq of ePCs cell fraction.

Under hyperoxia, two of the 13 genes, *POSTN and CCDC102B* were expressed significantly higher in eMSCs compared to eTAs. In comparison, *CNTN1* was enriched in eTAs (Figure 5.8 A).

Under physoxia, five out of thirteen genes were enriched in eMSCs: *POSTN, GUCY1A2, ANGPT2, EPHA3,* and *F2RL3*. *CNTN1* expression which was higher in eTAs was reversed under physoxia condition (Figure 5.8 B).

When gene expression was compared between eMSCs under hyperoxia and physoxia, five genes were upregulated: *POSTN, GUCY1A2, PDE1A, ANGPT2* and *EPHA3*. *CCDC102B* was enhanced in the eMSCs fraction (Figure 5.9 A). Similarly, when comparing TAs, under physoxia, *OLFML2A* was enriched, and *CNTN1* was increased under hyperoxia (Figure 5.9 B). Considering all the data together, under hyperoxia, eMSCs and eTAs were almost similar as only two genes were differentially expressed. Under physoxia, the difference between eMSCs and eTAs widens as five of the perivascular niche genes are induced in eMSCs. Even within eMSCs cultured in hyperoxia and physoxia, physoxia increase the expression of perivascular niche genes. This might be due to perivascular niche has a low oxygen concentration, and MSCs reside in a low oxygen niche to maintain their naïve state.

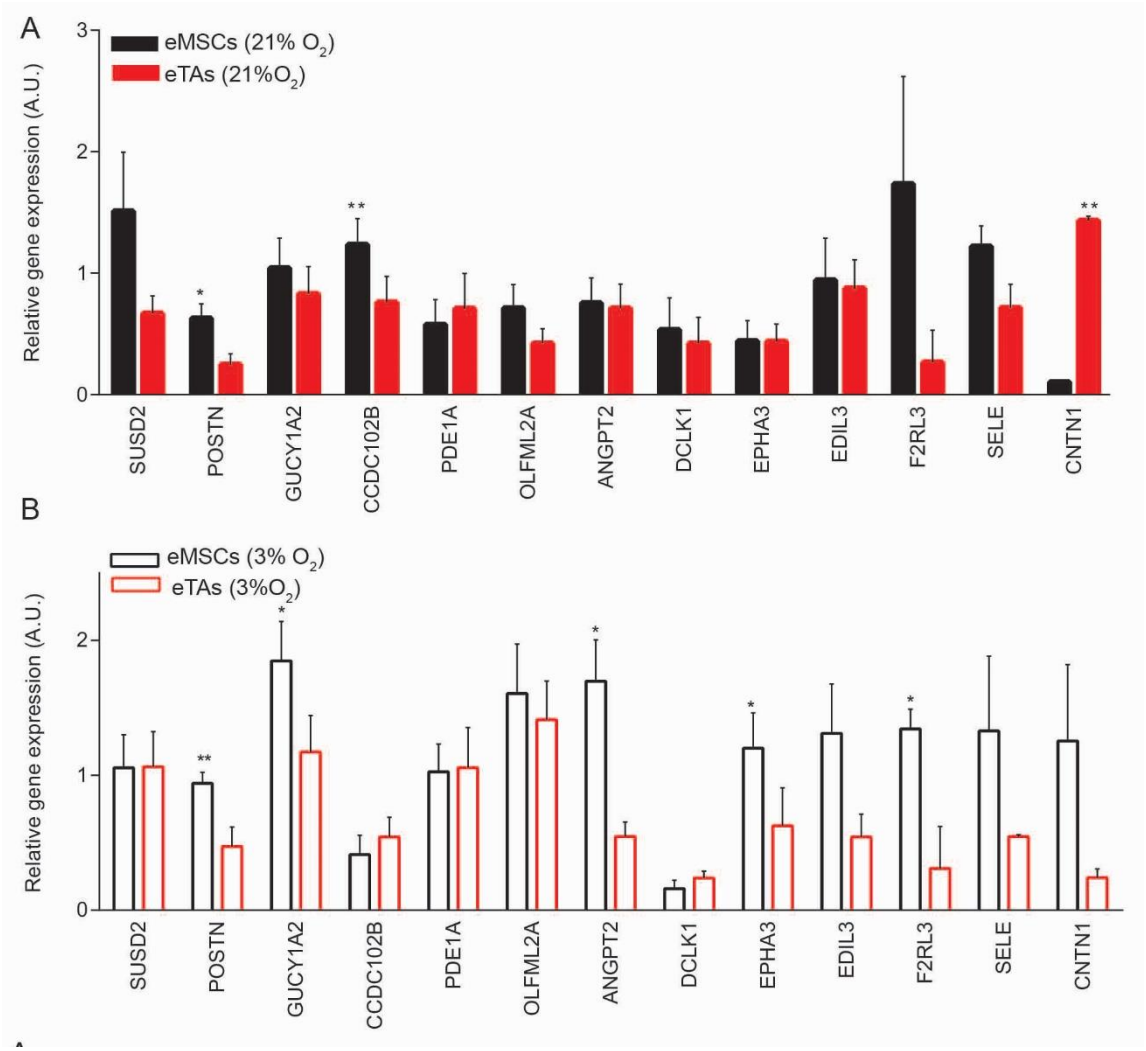


Figure 5.8. Oxygen-dependent gene expression in eMSCs and eTAs under 21%O_2 or 3% O_2.

Magnetically isolated ePCs and EnSCs cells were subjected to colony forming unit assays under 21% oxygen and 3% oxygen. At the end of 15 days, RNA was harvested from eMSCs, and eTAs cells and mRNA transcript expression of 13 differentially expressed genes were analysed in six paired biological replicates, in triplicate, using RT-qPCR. A. Differential gene expression between eMSCs and eTAs cells cultured under 21% oxygen (n=6). B. Differential gene expression between eMSCs and eTAs cells cultured under 3% oxygen (n=6). ** indicates $P < 0.01$. * indicates $P < 0.05$. Data represent mean ± SEM.

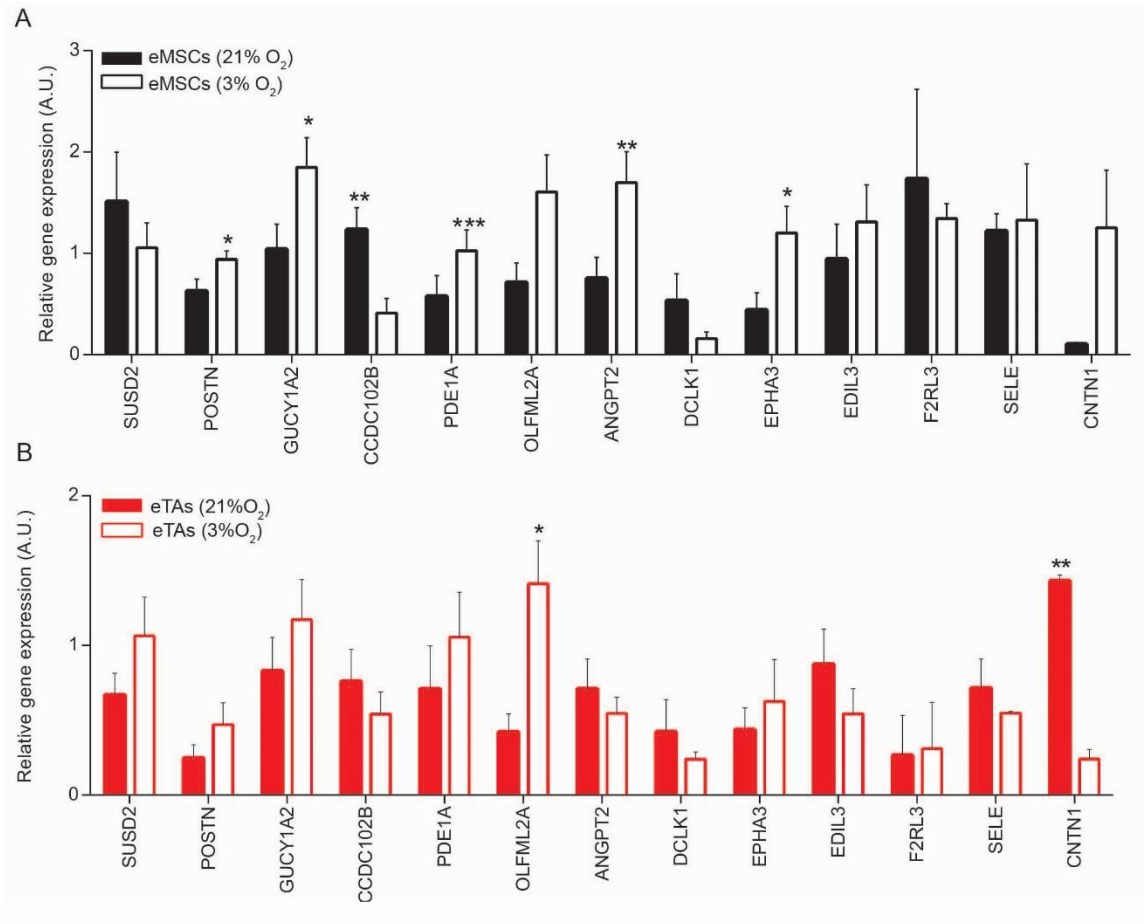


Figure 5.9. Oxygen-dependent gene expression in eMSCs or eTAs under varying oxygen conditions.

Magnetically isolated ePCs and EnSCs cells were subjected to colony forming unit assays under 21% oxygen and 3% oxygen. At the end of 15 days, RNA was harvested from eMSCs, and eTAs cells and mRNA transcript expression of 13 differentially expressed genes were analysed in six paired biological replicates, in triplicate, using RT-qPCR. A. Differential gene expression between eMSCs cells cultured under 21% and 3% oxygen (n=6). B. Differential gene expression between eTAs cells cultured under 21% and 3% oxygen (n=6). *** indicates $P < 0.001$. ** indicates $P < 0.01$. * indicates $P < 0.05$. Data represent mean ± SEM.

5.2.6 Angiogenic potential of eMSCs

To check the differentiation potential of eMSCs and eTAs, I investigated the ability of these cell types to form tubules when seeded on Matrigel in the presence of angiogenic media. CFU-F from three mid-luteal biopsies separated into ePCs, and EnSCs cell fractions were cultured for 15 days, cells were trypsinized, and 50,000 cells were seeded on Matrigel in a 96-well plate. Following overnight incubation, wells were examined for the presence of tubules and imaged using a phase contrast microscope. Images were analysed using ImageJ for the area covered by either tubes or cells. eMSCs formed tubules which covered significantly larger areas on the Matrigel compared to eTAs. No tubes were formed by eTAs (Figure 5.10).

5.2.7 Formation of ERBs

While analysing the angiogenic potential of eMSCs and eTAs, I fortuitously found the formation of tissue structures in most of the wells with eMSCs and several from eTAs. The most common structure is shown in Figure 5.11 A, was a three-lobed triangular structure. Because these structures showed rapid generation overnight, we coined the term ‘endometrial regenerative bodies’ or ERBs. These structures formed when cultured on Matrigel and in the presence of the angiogenic media. These structures were probed for endothelial marker von Willebrand Factor (vWF), which came out negative (result not shown). The ERB were fixed, processed and sectioned at 5 µm thickness and stained with hematoxylin and eosin (H & E). H & E staining of the structure revealed that the section resembled the stained biopsy section of the endometrium (Figure 5.11 B).

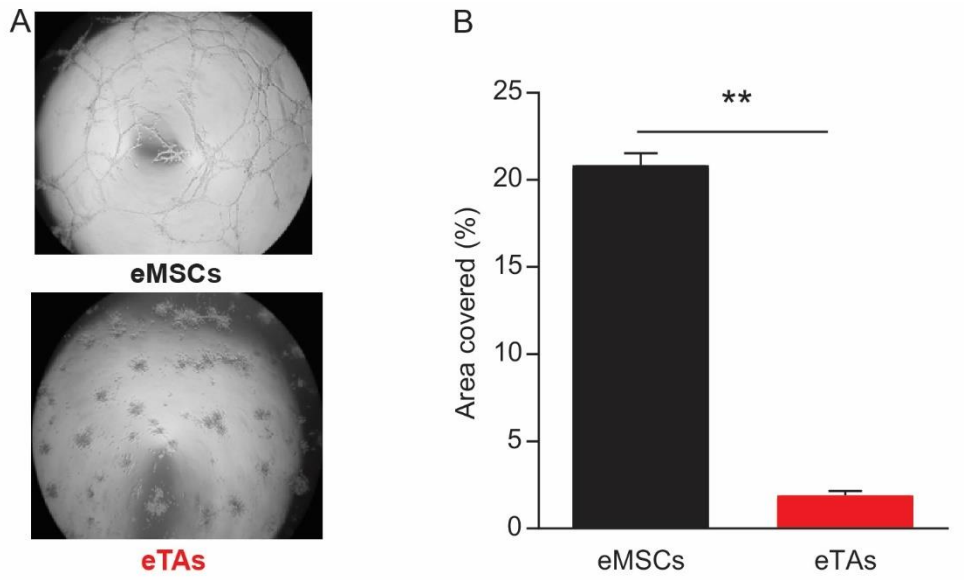


Figure 5.10. Angiogenic assay of eMSCs and eTAs cells.

After CFU-F, eMSCs and eTAs cells were trypsinized, and $5x10^4$ cells were seeded on Matrigel with angiogenic media and cultured overnight for angiogenic assay. A. Representative wells of the angiogenic assay from eMSCs and eTAs cells. Imaged using phase contrast microscope (magnification: ×4). B. The area covered either by tubes or cells were measured in percentage using Adobe Photoshop. The assay was carried out in 3 biological replicates in triplicates. ** indicates $P < 0.01$. Data represent mean ± SEM.

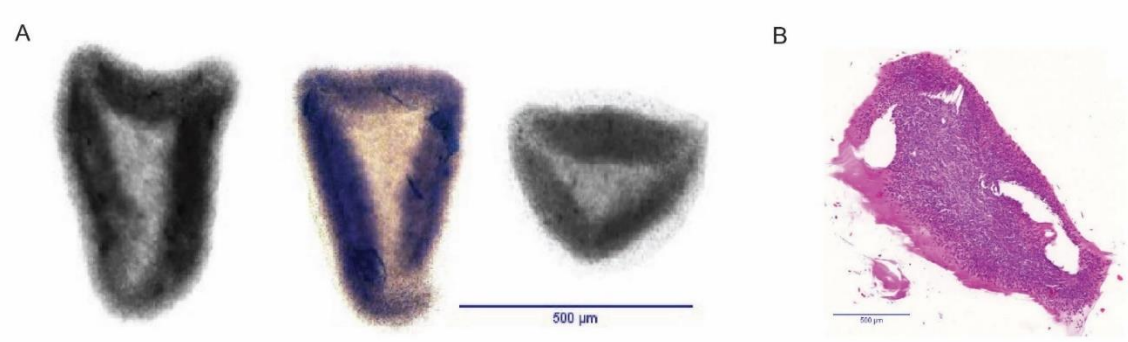


Figure 5.11. Gross morphology of Endometrial Regenerative Bodies (ERBs).

Most of the eMSCs and few of eTAs cells formed structures termed ERBs. A. Morphology of ERBs formed from either eMSCs or eTAs cells with three-lobed triangular structures, considered as optimal ERBs. Imaged using phase contrast microscope (magnification: ×4). B. Sagittal section of ERB stained with Hematoxylin and eosin (H&E) (magnification: ×4).

5.2.8 Time-lapse imaging

While characterising the ERBs, the striking observation was their rapid formation overnight. To delineate the formation of ERB, time-lapse imaging was carried out every 5 minutes for 48 hours with 5% CO_2 and 37 ^{o}C. The images over 48 hours suggested that the stages of ERB formation can be separated into 3 major steps: (i) during 0-9 hours, there was an initial rapid contraction of cell layer; (ii) through 12-21 hours, further compaction in the peripheral edges; (iii) 22-48 hours, circular structure became more organized into the final triangular three-lobed structure (Figure 5.12).

5.2.9 ERB formation from live and formalin fixed

For further characterization, I wanted to confirm that the formation of ERBs was not due to the contraction of Matrigel. To evaluate this, three biological replicates of eMSCs were harvested from CFU-F plates and counted. Cells were either fixed with formalin or left untreated. The untreated and formalin fixed eMSCs were subjected to ERB formation assay on Matrigel in the presence of angiogenic media. 100% of untreated cells formed structures whereas there was no ERB formation in the formalin fixed eMSCs. In formalin-fixed cells, there was no clumping or cell aggregation either. This shows that the ERB formation is due to the movement of cells of the surface of Matrigel rather than the shrinkage of Matrigel (Figure 5.13).

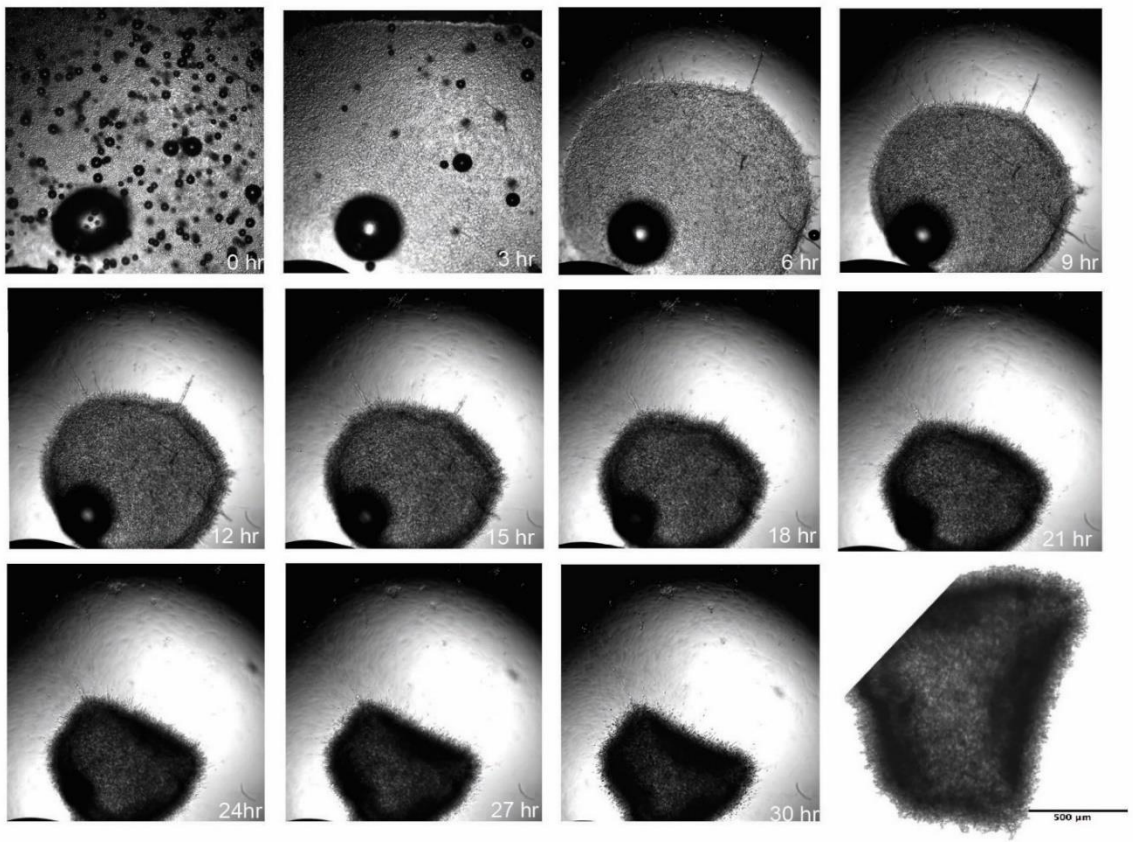


Figure 5.12. Time-lapse imaging of ERB formation.

ERB formation was recorded for 48 hours using time-lapse imaging microscope to show a representative change through time. Differential interference contrast (DIC) microscopy images were taken every 5 minutes. Representative images obtained at 3-hour intervals are shown.

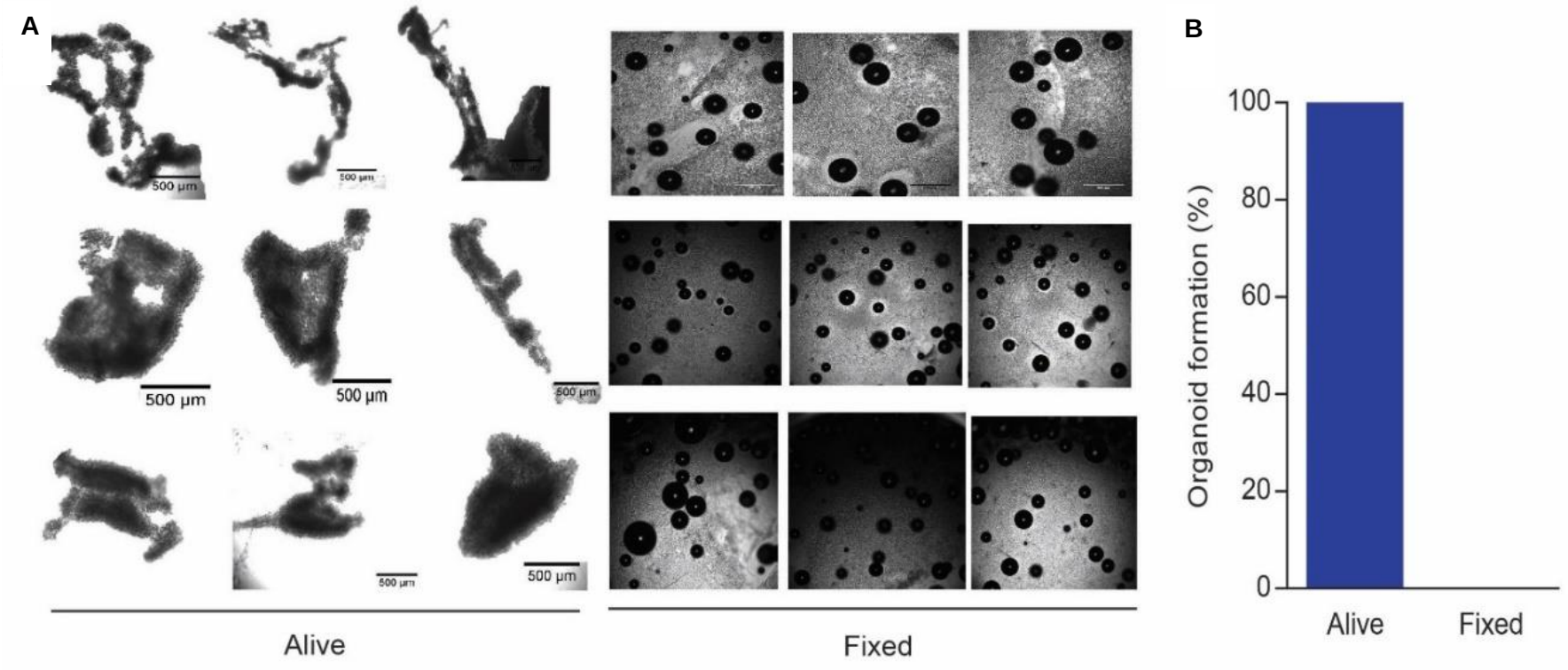


Figure 5.13. ERB formation from live and formalin fixed eMSCs cells.

eMSCs cells were trypsinized, counted, and a portion was fixed in 100 µl formalin for 10 min and the other portion of cells without any treatment, which served as a positive control. These cells were seeded on Matrigel in the presence of angiogenic media. A. representative images of structures formed from live eMSCs cells and no structure in the fixed eMSCs population. N=3 in triplicates. B. Percentage of ERB formation was calculated from the number of structures formed.

5.2.10 Analysis of ERB formation from endometrial stromal cell subpopulation

During initial formation of ERBs, there were few structures formed by eTAs. I wanted to analyse the potential of other cell populations within the endometrium to form ERBs when seeded on Matrigel in the presence of angiogenic media. For this experiment, the cell types used were: eMSCs, eTAs, ePCs, EnSCs and unselected endometrial stromal cells (uESCs). Different cell types were seeded, checked for the formation of ERBs the following day and imaged using a phase contrast microscope. Comparatively, eMSCs formed more ERBs (67%), followed by eTAs (15%), whereas there were no structures formed by neither ePCs and EnSCs cells nor endometrial stromal cells (Figure 5.14). Overall ERB formation potential is highest in eMSCs, lesser in eTAs and absent in the other cell types.

5.2.11 Effect of oxygen concentration on ERB formation

Since the concentration of oxygen had an impact on size and number of colonies formed both in eMSCs and eTAs, I assessed whether it had any impact on ERB formation or size. For this experiment, CFU-F was carried out in physoxia and hyperoxia. Following 15 days of culture, eMSCs were harvested and seeded for ERB formation assay, and the physoxia treated eMSCs were moved to hyperoxia for ERB formation assay and vice versa (Figure 5.15 A). The following day, the ERBs were imaged using phase contrast microscope and area measured. eMSCs that were cultured and ERB assay in hyperoxia had a significantly larger area compared to those cultured entirely in physoxia (Figure 5.15 B). The activation of eMSCs is vital under hyperoxia which is crucial for the formation of ERBs whereas under physoxia these cells are in a naïve state.

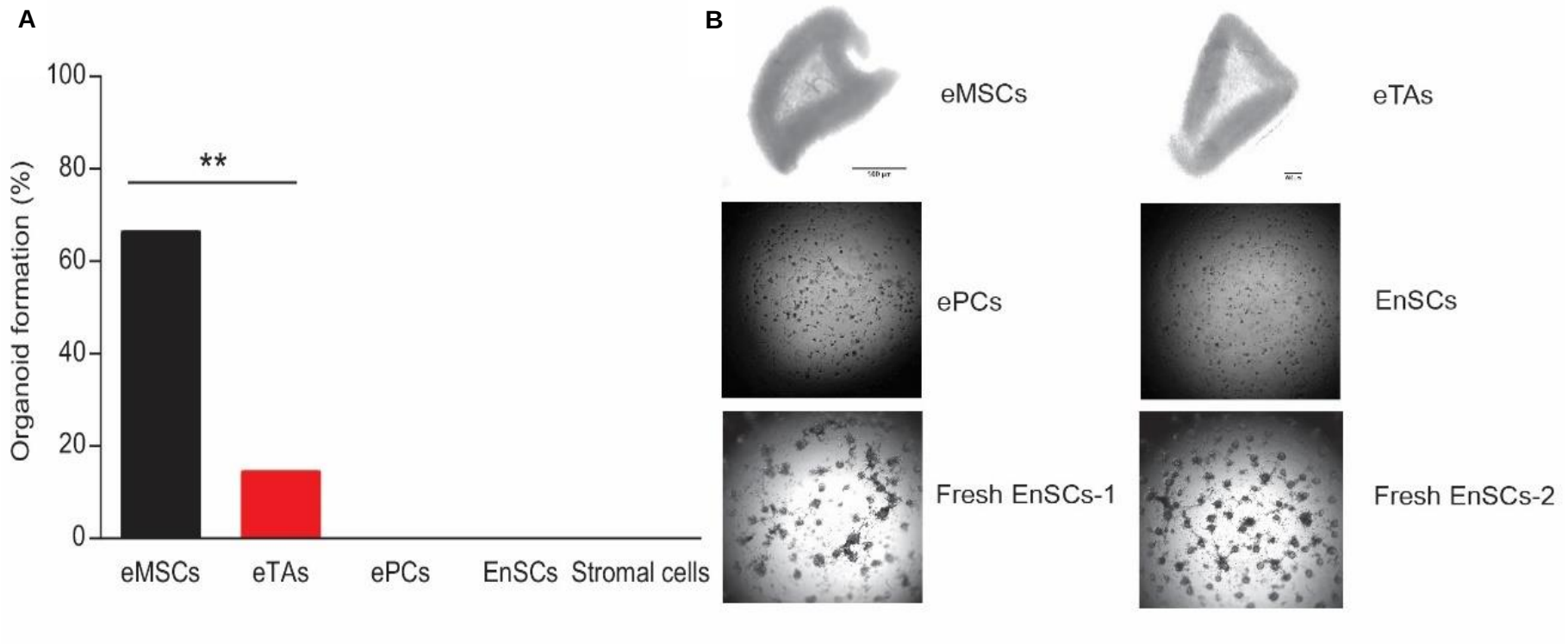


Figure 5.14. ERB formation within an endometrial stromal subpopulation.

To identify which population of cells are capable of forming ERBs, different population were analysed- (i) paired eMSCs and eTAs cells (n=9) (ii) magnetically selected ePCs and biopsies (n=6) (iii) unselected population (n=6). A. Representative images of ERBs formed from (i) eMSCs, and eTAs population and cells without any structures from (ii) magnetically selected ePCs and EnSCs cells and (iii) unselected population. B. Percentage of organoids formed was calculated based on the number of structures formed. ** indicates $P < 0.01$. Data represent mean ± SEM.

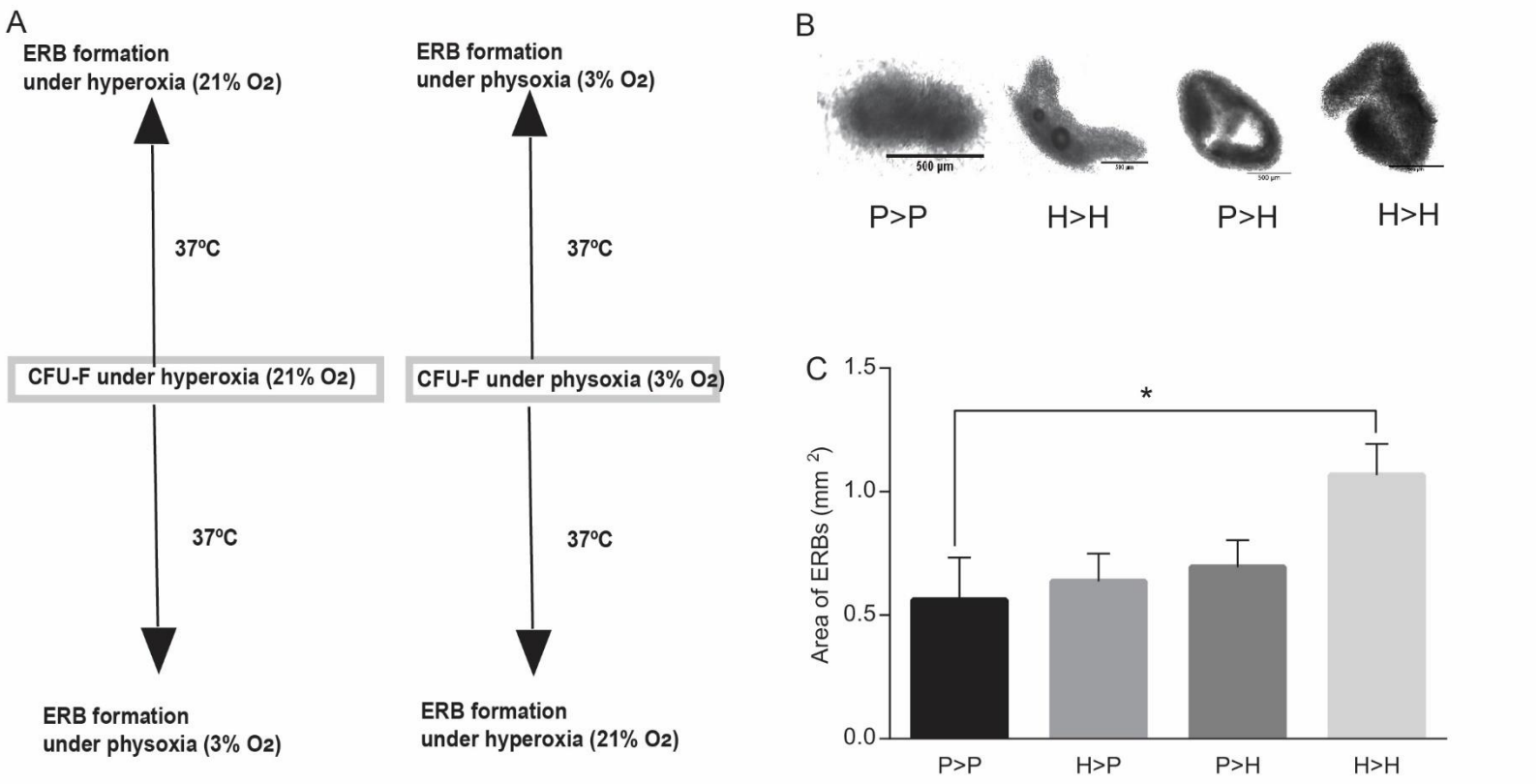


Figure 5.15. Effect of oxygen on ERB structure.

To analyse the effect of oxygen of ERB structure, eMSCs and ERB were cultured in varying oxygen concentrations- (i) P > P; (ii) H > P; (iii) P > H and (iv) H > H. A. Schematic diagram of the experimental plan. B. Representative images of structures formed under varying oxygen concentrations. n=3 in triplicates. B. area of structures formed was measured using ImageJ. * indicates $P < 0.05$. Data represent mean ± SEM. Data represent mean ± SEM. P denotes Physoxia; H denotes Hyperoxia

5.2.12 Re-epithelialization of ERB

As the morphology of ERB was similar to endometrial stromal tissue, I evaluated whether these structures could support endometrial epithelial cells (EECs). For this assay, ERBs were formed using eMSCs as described previously in Matrigel and 10^4 human endometrial epithelial cells were seeded. This ERB and epithelial cell coculture were incubated at 37^{o}C with 5% CO_2 overnight. The following day, ERBs with cocultured epithelial cells were fixed and stained using Vimentin, a marker for stromal cells and Cytokeratin 18, a marker for epithelial cells. Visualisation using a spinning disc microscope showed a single layer of epithelial cells formed on the ERB tissue structure (Figure 5.16). Therefore, I could demonstrate that ERB structure can be made more complex with the addition of other cell types relevant to the human endometrium.

5.2.13 Effect of agitation on cocultured ERBs

Lancaster and Knoblich (2014) showed that brain organoids were short lived due to a shortage of gas supply to the centre of the organoid and can be maintained *in vitro* for a longer period using agitation. Similarly, I hypothesised that agitation could enhance the lifespan of ERB. For this experiment, ERBs were formed as described earlier with and without coculture of EECS, overnight. The following day, ERBs were removed carefully from Matrigel into a new 96-well plate, which contained specialised organoid media. These ERBs and cocultured ERBs were imaged using a phase contrast microscope and placed on a plate shaker which was agitated at 250 rpm for over two days, along with controls without any agitation. After two days, agitated ERBs and cocultured ERBs along with controls were imaged. Static and agitated ERBs maintained the structure irrespective of the agitation. This showed that ERBs

could be removed from Matrigel and maintained in liquid media, without disruption of the structure.

ERBs cocultured with EEC, both under static and agitated conditions, formed a smooth surface around the edges, whereas it was not observed in those without EECs. (Figure 5.17).

5.2.14 Morphology of decidualized ERB

For determining whether the ERBs function similar to *in vivo*, ERBs were differentiated using cAMP and MPA for eight days and controls were maintained without any treatment. Following differentiation, the morphology and gene expression were analysed. After six days of decidualization, ERBs were fixed and sectioned. Undecidualized stromal cells have a fibroblast-like appearance with elongated and indented nuclei, whereas decidualized cells have rounded nuclei, which is similar to *in vivo* H&E stained sections (Figure 5.18).

For gene expression, ERBs were untreated or decidualized for 8 days. These ERBs were harvested for RNA, cDNA was synthesised and analysed for putative decidual markers; *PRL* and *IGFBP1* in 3 different paired ERBs.

PRL and *IGFBP1* mRNA level expression were more than 100-fold induction in response to cAMP and progesterone treatment, compared to the control (Figure 5.19 A, B).

Since PR regulates decidual markers, I analysed the mRNA expression *PR* and *ER* in response to decidualization for over eight days. The expression of *ER* reduced whereas the expression of *PR* increased, in response to decidualization (Figure 5.19). Taking together, the morphology change and gene expression in response to cAMP and MPA, ERBs respond in the similar way to stromal cells respond to decidual cues.

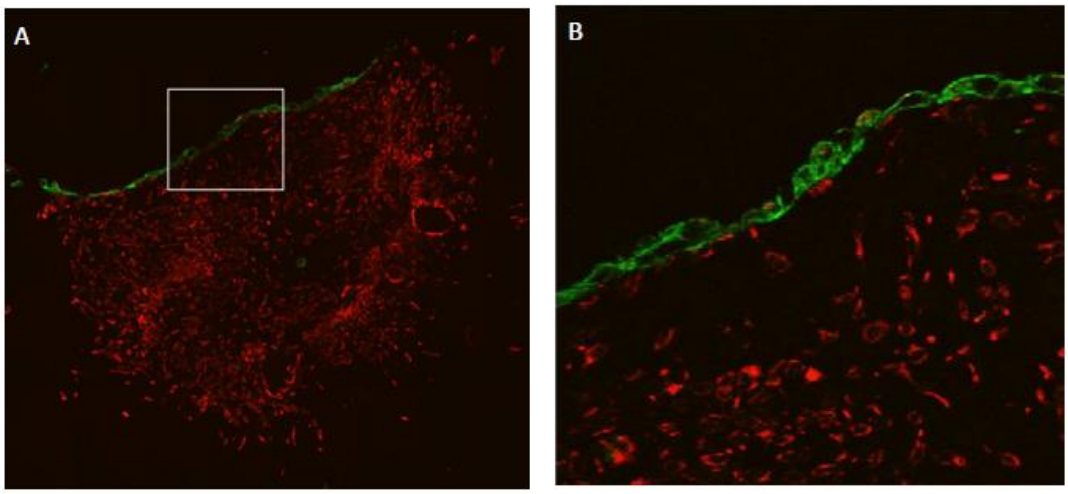


Figure 5.16. Immunohistochemistry of ERBs co-cultured with endometrial epithelial cells (EECs).

ERBs were formed from eMSCs cells (1×10^5) overnight, and primary EECs (1×10^4) were co-cultured for 24 hours. Co-cultured ERBs were fixed, sectioned and stained for Vimentin (red-eMSCs) and Cytokeratin 18 (green-EECs). These sections were imaged using spinning disc microscope. A. A spinning disc stitched image at magnification: ×10. B. A spinning disk image at 40x.

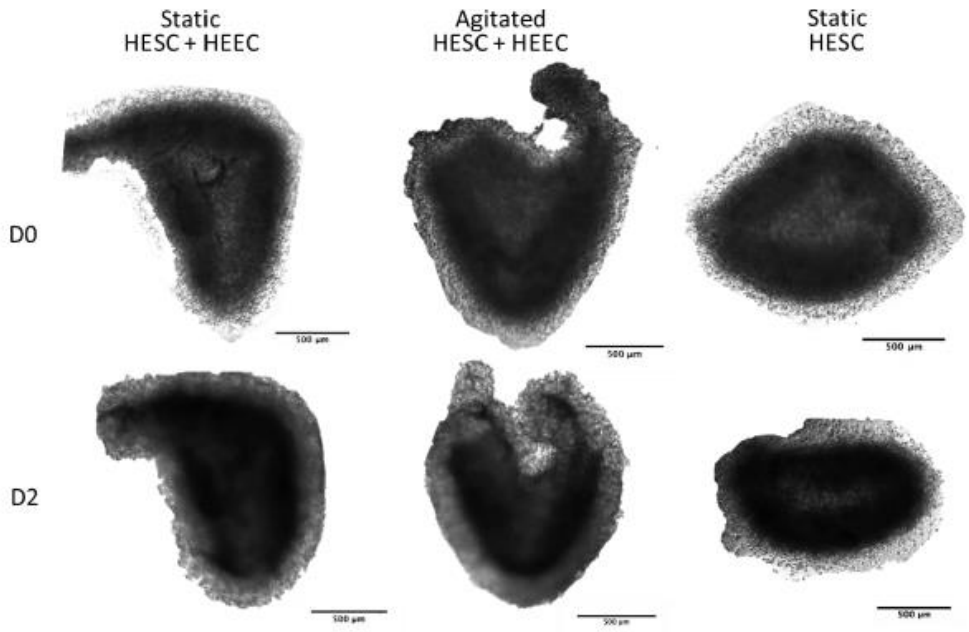


Figure 5.17. Effect of agitation on ERBs co-cultured with EECs.

ERBs made of eMSCs, and ERBs co-cultured with EECs were cultured at the standard static condition and imaged using phase contrast microscope at magnification: ×4 (D0). One of the ERB co-cultured with EECs was agitated at 250 rpm for 48 hours and re-imaged at magnification: ×4 (D2).

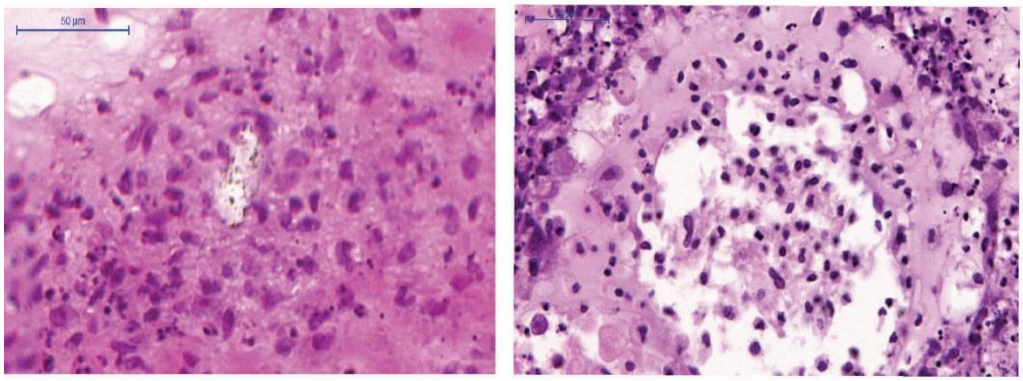


Figure 5.18. Hematoxylin & Eosin staining sections of untreated and decidualized ERBs.

ERBs were treated with 2% DCC or 2% DCC in the presence of cAMP + MPA for six days. The staining shows that undecidualized cells (D0) have a fibroblast-like appearance with elongated and indented nuclei, whereas the D6 cells have rounded nuclei, which is similar to *in vivo* H&E stained decidualized sections.

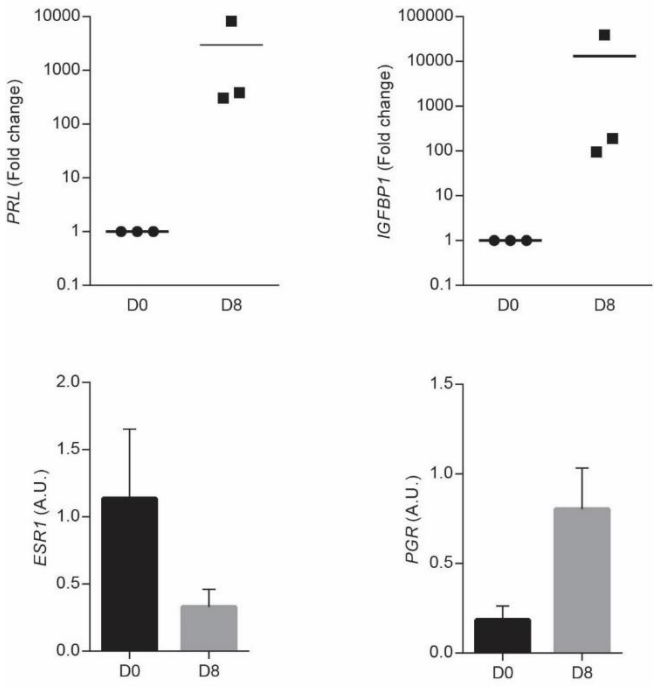


Figure 5.19. Induction of decidual markers and changes in nuclear receptors.

A. Classical decidual markers PRL and IGFBP1 were induced upon decidualization for eight days. The induction is shown as fold change. Three biological replicates in triplicate. B. Nuclear receptor ESR1 responsible for oestrogen binding went down whereas progesterone binding PGR increased with decidualization treatment.

5.3 Discussion

5.3.1 Regeneration potential of the human endometrium

Human endometrium undergoes dynamic changes through the reproductive life of a woman to meet the demand of embryo implantation and highly invasive placenta (Evans et al., 2016). The endometrium undergoes around 400 menstrual cycles with a high level of cellular turnover and in turn the massive level of tissue regeneration. It undergoes controlled tissue remodelling and grows from 0.5-1mm into massive 7 mm thick tissue following menstrual shedding (McLENNAN and RYDELL, 1965). Behind this regeneration, there is a rapid angiogenesis that occurs under strict regulation. During embryogenesis, the endometrium is formed by the fusion of the mucosal lining of paramesonephric tubes, is composed of two major zones: functionalis and basalis. The basalis layer has glands, stroma, supporting vasculature and lymphoid aggregates. Stem/progenitor cells are located in the basal layer and progenitor cells multiply rapidly into transient amplifying cells eventually moving into the functional layer participating in the active regeneration and remodelling of the endometrium (Okulicz et al., 1997).

5.3.2 ePCs gene expression

ePCs have been identified to be pericytic. ePCs are localised to basalis layer and around the vessels in the functionalis layer. Transcriptomic analysis of cultured ePCs cells confirmed that they have a perivascular phenotype. The notable perivascular markers that are enriched in culture are *NOTCH3, MCAM (CD146), AOC3, ELN, and MYH11*. Pericytic or MSC related genes that were enriched in cultured ePCs cells are *NT5E* (CD73), *ENG* (CD105), *ITGA5* (CD49e), *ALCAM* (CD166), *CD44* (CD44), *ITGB1* (CD29), *THY1* (CD90) and *PDGFRB* (CD140b). Meanwhile, secretome analysis of decidualized ePCs and EnSCs cells show that they elicit a divergent

decidual response, a proinflammatory response by ePCs and an anti-inflammatory response by EnSCs cells (Murakami et al., 2014).

In this chapter, the transcriptomic characterization was carried out for magnetically sorted fresh ePCs and EnSCs cells. Within the EnSCs population, the expressed gene signature is widely related to an immune cell population. This is because the depletion of immune cells was not carried out before the RNA sequencing. Though there was a strong immune signature within EnSCs population, there was a tight gene signature that is differentially expressed only in ePCs population. The genes that were differentially expressed in ePCs population are *ANGPT2, CCDC102B, CNTN1, DCLK1, EDIL3, EPHA3, F2RL3, GUCY1A2, OLFML2A, PDE1A, POSTN, SELE, and SUSD2.*

ANGPT2 and MMPs secreted by uNK cells are vital in spiral artery remodelling during pregnancy (Robson et al., 2012). Following menstruation, in the presence of VEGF, ANGPT2 secreted by perivascular cells might be involved in the blood vessel regeneration. *ANGPT2* is competitive and mostly antagonistic to *ANGPT1;* it destabilises blood vessel walls and vessel regression in the absence of VEGF (Maisonpierre et al., 1997). In the presence of VEGF, it promotes vessel sprouting and endothelial cell migration (Lobov et al., 2002, Pourjafar et al., 2016). *ANGPT2* is expressed by endothelial cells in some physiological conditions such as wound healing. It is upregulated under physoxia by growth factors including VEGF and bFGF (Mandriota and Pepper, 1998, Okada et al., 2015).

Glycosylphosphatidylinositol (Zhang et al.) anchored neuronal membrane protein, CNTN1 is an adhesion molecule (Mikami et al., 2009) and a key ligand and activator of *NOTCH1 (Schweitzer et al., 2007).* Studies show that it plays a major role in adhesion, migration, and invasion in thyroid cancer and upon knockdown inhibits

cyclinD1 (Shi et al., 2015). CNTN1 might be playing a similar role in endometrial cells regulating cellular physiological processes.

DCLK1 has been previously reported to be involved in neuroblast proliferation, migration and differentiation (Shu et al., 2006). *DCLK1* has a domain that can bind tubulin and enhance microtubule polymerization. *DCLK1* plays a role in stem cell fate decision in a 3D environment based on the onset of stress due to stiffening of the polyisocyanopeptide-based hydrogels (Das et al., 2016).As ePCs are located around blood vessels DCLK1 could contribute to proliferation, migration during the vessel remodelling following embryo implantation.

EDIL3, a glycoprotein, secreted by endothelial cells and is regulated by physoxia or vascular injury. During angiogenesis, it has been implicated in vascular remodelling (Choi et al., 2008). It promotes endothelial cell adhesion and migration (Penta et al., 1999).Following menstruation EDIL3 might be secreted by endothelial cells.

EPHA3, an ephrin family protein member, has a major function in tumour progression and expressed in the vascular rich regenerating human endometrium. This is gene is controlled by HIF1A and is involved in cell contraction and migration (To et al., 2014).

F2RL3 or *PAR4,* a member of protease-activated receptor (PAR) family, when activated follows G-protein signalling (Gq) and cause a calcium mobilisation. *F2RL3* promotes the activity of some calcium-regulated kinases and phosphatases that control *PAR4* induced cellular responses such as platelet activation and vascular remodelling. PAR4-$G_{12/13}$ interaction causes Rho activation that controls cytoskeletal responses in vascular smooth muscle cells and endothelial cells (French and Hamilton, 2016).

Guanylate Cyclase 1, catalyses the conversion of guanosine triphosphate (GTP) to 3',5'-cyclic GMP and pyrophosphate. The protein encoded by this gene *GUCY1A2* is an alpha subunit of this complex, and it interacts with a beta subunit to form the guanylate cyclase enzyme and activated by nitric oxide (RefSeq, 2012).

OLFML2A is a regulator that mediates smooth muscle differentiation process through growth factor, TGF-β. Smooth muscle differentiation is a significant process in vasculogenesis and angiogenesis (Shi et al., 2014).

PDE1A regulates vascular smooth muscle growth by maintaining the homoeostasis of cyclic nucleotides via hydrolysis of cAMP or cGMP. This has a major function in the transformation of vascular smooth muscle cells from contractile to synthetic morphology, following biological/mechanical injury or *in vitro* culture. This conversion gives the vascular smooth muscle cells the capacity to migrate, proliferate and to produce extracellular matrix proteins (Nagel et al., 2006).

Periostin, a matricellular protein that is activated by tissue damage and repair. *POSTN* activates *PI3K/Akt* pathway that regulates several functions such as cell survival, proliferation, migration and wound healing (Eun et al., 2010).

Cell adhesion molecules such as endothelial selectins (*SELE*) are expressed at the vascular stem cell niche, to which stem/progenitor cells adhere. *SELE* is usually expressed by endothelial cells at the site of inflammation, providing a way for inflammatory cells to adhere and migrate towards the site of inflammation or injury. *SELE* is crucial in stem cell niche, and it promotes stem cell proliferation (Winkler et al., 2012).

Data mining for the perivascular niches GEO database showed that five out of thirteen genes were expressed at a higher level during proliferative phase compared to other phases. A recent study characterised endometrial stem and progenitor population in

during three phases of the menstrual cycle and different layers of the endometrium. This study warrants that the self-renewal capability of stem cells is high during menstrual phase followed by proliferative phase (Shan et al., 2016). In this study, the higher expression of few of the genes of perivascular niche during proliferative phase (GEO profile) shows that the activation of a niche for regeneration of the functionalis layer following menstruation.

A similar study by Spitzer et al. (2012) compared transcriptomics of various cell populations within the human endometrium. In their comparison of eMSCs (MCAM (CD146^{+}/*PDGFRβ*$^{+}$) with endometrial stromal fibroblast populations (MCAM (CD146 $^{-}$/*PDGFRβ*$^{+}$), there were 762 significantly differentially expressed genes: 378 up-regulated and 384 down-regulated. Out of 378 upregulated genes, some genes were comparable to the list of genes enriched in the perivascular population. The genes are *PDE1A, ANGPT2, GUCY1A2, DCLK1, CCDC102B, EPHA3 and OLFML2B*, a member of Olfactomedin family.

Another study by Barragan et al. (2016) compared FACS isolated eMSCs and endometrial stromal fibroblasts (Gomaa et al.). In this population, when compared to MACS isolated ePCs and EnSCs cell population, the genes that were upregulated in FACS isolated eMSCs were: *ANGPT2, CCDC102B, DCLK1, PDE1A, GUCY1A2, OLFML2A,* and *SUSD2. CNTN1, EDIL3,* and *POSTN* were among the genes that were downregulated. This shows that these genes that are upregulated in ePCs cells have a robust gene expression irrespective of the marker or method of isolation.

5.3.3 ePCs functional characterization

As most of the differentially expressed genes within ePCs population play a role in proliferation, migration, contraction and are induced by physoxia, experiments were set out to analyse whether there are any differences in the functional characteristics

of ePCs and EnSCs cells. From the experimental results, it is clear that there is a divergence between perivascular ePCs and non-perivascular EnSCs populations, not only at gene level but also in functional level.

In the case of ePCs, there is more proliferation and migration compared to EnSCs cells. This might be because stem cells can proliferate in response to injury or wound healing (Caplon, 2005). ePCs cells have more clonogenic potential compared to EnSCs cells, which shows that eMSCs are enriched within the ePCs population (Murakami et al., 2013a). Previous studies show that eMSCs have migratory properties and migrate in a controlled manner towards chemotactic signals such as bFGF, IL-6, SDF-β and VEGF (Schmidt et al., 2006). Use of bFGF as a chemoattractant demonstrated the enhanced migration of ePCs cells in comparison to EnSCs cells.

5.3.4 ePCs oxygen consumption

Oxygen consumption of ePCs cells was lower when were compared to EnSCs cells. Energy metabolism studies analysed eMSCs on their metabolic profile and found that during proliferation and differentiation, they consume glucose mainly and produce lactic acid (Wang et al., 2005, Grayson et al., 2006, Mischen et al., 2008, Follmar et al., 2006). Under standard laboratory oxygen conditions, eMSCs consume glucose and produce lactic acid, which is termed as 'Warburg effect.' When cultured under hypoxic condition, the rate of glycolysis increases, which is termed as 'Pasteur effect' (Krebs, 1971).

Mesenchymal stem cells stay in a hypoxic niche, where the oxygen concentration ranges from 2-9%. Levels of oxygen determine the stem cell fate regarding differentiation and proliferation, regulated through *HIF1-α*. Some studies show that MSCs maintained in hypoxic conditions rather than laboratory oxygen concentrations

improve growth kinetics, gene stability and expression of chemokine receptors (Haque et al., 2013). Since the CFU-F is a method for quantification of stem cells, we used this method to study the effect of oxygen concentration on eMSCs and eTAs. The results reveal that physoxia has a significant increase in colony size and efficiency irrespective of cell types.

5.3.5 Effect of low oxygen concentration on eMSCs and eTAs

I wanted to analyse the perivascular gene expression pattern in eMSCs and eTAs under varying oxygen concentrations and also to check whether the near *in vivo* conditions had any effect on gene expression pattern. I have demonstrated that physoxia maintained the perivascular niche gene expression in a more robust way in eMSCs, compared to eMSCs maintained in normal laboratory oxygen condition. This result supports the fact that the low oxygen concentration or physiological oxygen concentration can improve the genetic stability of cells in culture (Haque et al., 2013).

5.3.6 Angiogenic potential of eMSCs

We have demonstrated the angiogenic potential of eMSCs by using Matrigel tube formation assay. For this assay, normal DMEM media was substituted with Medium 200, an angiogenic culture medium. The results showed that eMSCs can form tubes on the surface of Matrigel whereas there was no tube formation in eTAs. This demonstrates that eMSCs could reorganise on Matrigel and differentiate into an angiogenic lineage. Tube formation is possible as stromal cells and endothelial cells have a mesenchymal origin. Previously, endometrial stromal cells were shown to form tube-like sprouting on a 3D fibrin matrix (Lebovic et al., 2000).

5.3.7 Plasticity in forming ERBs

Following angiogenic assay using eMSCs and eTAs on Matrigel with angiogenic induction media, we observed macroscopic tissue structures (ERBs) that were rapidly formed following 16-18 hours of incubation. Human endometrium displays rapid degeneration and regeneration with tightly regulated tissue remodelling that results in a healed, scar-free tissue and fully regenerated functionalis layer in 2 weeks, (Cousins, 2014), with significant angiogenesis that occurs in a routine, physiological basis (Girling and Rogers, 2005). A number of attempts have been made in the past to create epithelial organoid culture including, intestinal epithelial organoids (Gjorevski et al., 2016), airway organoids (Tan et al., 2017) , salivary gland spheroids (Shin et al., 2016) and several attempts in the case of endometrial epithelial organoids (Bläuer et al., 2005, Bläuer et al., 2008, Gargett et al., 2009). The ERBs on Matrigel formed structures that are mostly three-lobed triangular structures, which were similar to structures that were formed by fibroblasts on Matrigel under the influence of TGF-β1 in relevance to wound healing (Fan et al., 2015).

We identified that frequency of ERB formation is higher in eMSCs and to an extent in eTAs. The formation of ERBs from eTAs partly might be attributed to the low volume of W5C5^{+} antibody utilised during magnetic isolation. Also, it should be considered that eMSCs are lineage precursors of eTAs as reported in a recent study (Barragan et al., 2016)Compared to other cell populations including unsorted stromal populations or MACS sorted ePCs and EnSCs cells, the ERB formation is more specific to the eMSCs population.

The shape of ERBs varied from three-lobed triangular structures to two-lobed structures and malformed structures. Similar variations in the organoid structures were reported previously in the case of cerebral organoids, where they have devised a classification system naming them suitable and sub-optimal organoids (Lancaster

and Knoblich, 2014a). Similar terminologies could be utilised to discriminate types of ERBs based on their structure.

5.3.8 ERB characterization

Time lapse imaging of ERBs overnight gave an insight into their formation, in which the cells initially form a sheet-like structure and followed by inward contraction of cells towards the centre. After a few hours, there was a proliferation of cells at the edges of the structure which then fold up around the edges, slowly transforming the circular structure into a three-lobed triangular structure. During the first hour of ERB formation, it can be observed that there is a movement of Matrigel, due to polymerization. We confirmed that this contraction does not result in ERB formation by seeding formalin fixed eMSCs and observed a lack of structure formation or movement of the cells.

5.3.9 Effect of oxygen concentration on ERB formation

Physoxia had a significant effect on size and area of clonogenic population of eMSCs and eTAs, in our experiments. Additionally, it can be concluded that eMSCs cultured for ERBs in normal laboratory oxygen conditions formed larger triangular three-lobed structures, whereas, the ERBs cultured in physoxia condition were malformed or smaller.

Organoids are self-organized structures derived from stem or progenitor cells that differentiate into complex tissue structures containing multiple cell types (Lancaster, 2014). We increased the cellular complexity of the ERB by culturing it with endometrial epithelial cells, to form an epithelialized structure. Epithelialized ERBs may be able to recapitulate human endometrium which could be used as a novel *in vitro* implantation model. Immunostaining following ERB re-epithelization showed that epithelial cells could line a layer of transformed eMSCs. It is important to achieve glandular formation within the ERB structure, which calls for a more complex culture method, to

authenticate ERB as an appropriate *in vitro* implantation model. Time lapse imaging of fluorescent tagged eMSCs and endometrial epithelial cells might shed light on spatial orientation and cell sorting within the organoid structure.

5.3.10 Effect of agitation

Lancaster and Knoblich (2014a) showed that agitation improved the nutrient and gas exchange to the inner core of cerebral organoid. We found that with agitation, the structures can be maintained for a shorter period, but this preliminary data is inconclusive, as longer time exposure of agitation is required to examine whether it has any specific impact on either structure or longevity of the ERB.

5.3.11 Reepithelization of ERBs

I aimed to increase the cellular complexity of the ERB, by the addition of human endometrial epithelial cells (HEECs) to re-epithelialize the tissue. This was done to develop the ERB into an organoid that is capable of recapitulating the human endometrium-like structure for the development of a novel implantation model. Immuno-staining of co-cultured ERBs shows a layer of epithelial cells lining a mass of eMSCs. This is promising for further expanding the cellular complexity of ERBs.

5.3.12 Functional differentiation of ERBs

Finally, I investigated whether the ERBs were capable of recapitulating endometrial biological functionality. I decided to decidualise the ERBs as it is indispensable implantation.

The results indicated that ERBs respond to hormonal actions of MPA and cAMP. The morphological transformation was like that of previously reported (Gellersen, B., et al., 2003). Further, the RT-qPCR confirmed the characteristic changes in gene expression for decidualized cells with the induced expression of decidual markers

PRL and *IGFBP1*. Similarly, PR and ER expression represent changes as expected during decidualization reactions.

Following menstruation, eMSCs play a major role in repair and reconstitution of the functional layer of the endometrium along with the rapid angiogenesis. Relative deficiency in eMSCs population results in perturbed proliferation followed by aberrant decidual response leading to implantation related issues. A recent study from our group compared eMSCs and eTAs population within the control (N = 28) and recurrent miscarriage group (N = 31) and demonstrated that there is about 41% reduction in eMSCs in recurrent miscarriage group (Lucas et al., 2015). This shows that deficiency of eMSCs might lead to recurrent pregnancy loss.

In this chapter, I have decided to focus on ePCs, a specific population from which eMSCs could be isolated. I have shown perivascular ePCs cells have a unique gene signature, and as a result, differ functionally from non-perivascular EnSCs cells. The ePCs population is rich in clonogenic cells, or eMSCs have higher propensity to prosper under lower oxygen concentrations and have higher angiogenic potential. The structures that were identified during an angiogenic assay, ERBs, is first endometrial stromal cell structure to be reported. eMSCs have more potential of forming ERBs and better tissue organisation when cultured under normal oxygen concentration. These structures can be made more complex by coculturing with epithelial cells and can be decidualized. Further, complex structural modifications of ERBs along with frequent nutrient and hormonal perfusion will facilitate to achieve a tailor-made, patient specific novel *in vitro* embryo implantation model that could be used to understand implantation related issues which will eventually aid in developing treatments. Three decades ago, the concept of endometrial stem/progenitor cells was proposed as a reason for the immense regenerative potential (Prianishnikov, 1978). Stem cell self-renewal and differentiation are associated with cell cycle advancement

that enables tissue homoeostasis (Pauklin and Vallier, 2014). Our recent study shows that human endometrial stromal cells have a distinct subpopulation of cells ranging from eMSCs, eTAs, ePCs, EnSCs, pre-senescent cells and senescent cells (unpublished data). The quality of eMSCs defines the nature of the estrogen-mediated proliferation, subcellular population and decidualization, leading to the success of pregnancy.

Stem cell properties are defined by the tissue-specific microenvironment (Fuchs et al., 2004, Scadden, 2014). The perivascular niche of human endometrium around the spiral arterioles consists of quiescent and active eMSCs, transient amplifying cells, and adjacent non-stem cells.

In this study, I demonstrate that there is a difference between ePCs and EnSCs at the transcriptomic level, *in vitro*. The genes that were differentially expressed in ePCs population are *ANGPT2, CCDC102B, CNTN1, DCLK1, EDIL3, EPHA3, F2RL3, GUCY1A2, OLFML2A, PDE1A, POSTN, SELE and SUSD2.* These genes are involved in various functions such as cell adhesion, cell contraction, metabolic processes and response to stimuli.

I have shown that the contractile capacity of perivascular cells is increased compared to that of non-perivascular cells. Contractile properties of these cells provide stability and functionality to blood vessels as well as regenerative capacity. (Wanjare et al., 2013).

My data also shows that oxygen consumption of ePCs was lower than EnSCs. This result is counterintuitive considering ePCs perivascular localisation. In the case of HSCs, a revised physoxia niche model of stem cells have been proposed, which suggests that the bone marrow cavity exhibits a higher oxygen concentration in arteriole-rich endosteal zones. However, the oxygen concentration is low in

perisinusoidal and periarteriolar regions, where the stem cells are proposed to reside (Nombela-Arrieta and Silberstein, 2014).

These results show that perivascular cells are adapted to their niche properties with enhanced contractile, adhesive and migratory properties and low oxygen consumption to maintain cell number and stemness.

Next, I characterised eMSCs, a subpopulation of ePCs with colony generating ability. The experimental results show that eMSCs and eTAs are enriched when cultured under physoxia conditions. Further, eMSCs could express more perivascular niche genes when cultured under physoxia. Thus, I report that physoxia condition is more supportive of eMSCs mimicking *in vivo* conditions.

eMSCs also formed 3D structures that resemble the uterine mucosa when cultured in Matrigel. These novel organoids, termed endometrial regenerative bodies (ERBs), epithelialize when co-cultured with endometrial epithelial cells and decidualize in response to differentiation cues.

Previous studies have attempted to achieve an *in vitro* embryo implantation model with various coculture methods with simplistic monolayer culture, or with cell inserts using immortalised/transfected cells to study implantation of trophoblast cells or hatched embryo (Holmberg et al., 2012) (Singh et al., 2010, Zhang et al., 2012) (Teklenburg and Macklon, 2009) (Bentin-Ley et al., 1994, Bentin-Ley et al., 1999, Bentin-Ley et al., 2000, Lopata et al., 2002) In this study, we are in the early stages of the development of a novel, rapidly generated *in vitro* implantation model. Hyperoxia supports the formation of ERBs as this likely recapitulates physiological conditions. These ERBs are capable of re-epithelization which means it does not require any inserts or coculturing. These structures are capable of decidualizing and express decidualization markers that are needed for embryo implantation.

ERBs could be a unique new resource to study an array of endometrial disorders tailor-made for patients. The results presented in my work show that ERBs could be manipulated to recapitulate near *in vivo* structure. The ultimate aim of the future work to develop this model to define mechanisms involved in embryo implantation failure.

My results show that ERBs express steroid hormone receptors (ESR1 and PGR). This raises the possibility of sequential treatment with oestradiol and progesterone leading to cyclic gene expression changes. Therefore, this model could be developed to investigate cell senescence and uterine natural killer cell infiltration further.

As this is a model for implantation, once the model is optimised with regard to cellular complexity and biological functionality, embryo implantation experiments can begin.

Chapter 6

6 General discussion

The human endometrium is considered a dynamic tissue which undergoes complex tissue remodelling and regeneration at least 400 times during a women's reproductive life. The endometrium consists of glandular and luminal epithelium within a cellular vascular stroma. The stromal cell population has previously been considered to be a homogeneous fibroblast cell population. However, in this thesis, I provide evidence that it is a combination of stem-, progenitor-, transit amplifying-, pre-senescent- and senescent-cells. (Unpublished work). The balance of these cell types is key in maintaining tissue homoeostasis. The ability of the endometrium to continuously complete cycles of shedding, regeneration and differentiation is attributable to endometrial stem cells which have unique high regeneration potential (Gargett et al., 2012a), compared to other adult stem cells (Somasundaram, 2016). Stem cells act to replenish tissue in the event of injury or insult. Cyclical menstruation is a physiological injury-like event, which is thought to provide a signal activating the stem cell niche, thereby recruiting stem cells for regeneration. The stem cell niche in the endometrium is localised perivascularly around the spiral arteries. This localisation ensures the provision of factors that are necessary for maintaining the number, as well as the quiescent nature of the stem cells (Murakami et al., 2014).

Decidual transformation of the endometrium starts around the spiral arteries. Perivascular cells and non-perivascular cells mount a diverse decidual response in the endometrial stroma. It is thought that decidualised perivascular cells secrete factors establishing a chemokine gradient, attracting trophoblasts for invasion (Murakami et al., 2014).

Decidual transformation of human endometrium equips the endometrium for the detection and selection of high-quality embryos and to discard poor quality embryos

(Gellersen and Brosens, 2014a). Fetal loss prevents maternal investment in poor quality embryos even before placental perfusion (Bielanska et al., 2002). Decidualization reprograms the endometrial stromal compartment at transcriptomic, proteomic and epigenetic levels. This reprogramming of the endometrium enables it to regulate trophoblast invasion, resist inflammatory and oxidative stress and allow embryo implantation (Gellersen et al., 2007).

This thesis has investigated the (i) robustness of clonal eMSCs /TA cell isolation from mid-luteal biopsies, (ii) assessment of the impact of tissue injury (i.e. biopsy) on endometrial clonal cell populations in different patient groups, (iii) validation of the link between aberrant decidualization and reproductive failure, (iv) In-depth characterization of eMSCs / ePCs cells.

In summary, I provide data showing that:

(i) Quantification of clonal (eMSCs/TA) cells in mid-luteal biopsies obtained in consecutive cycles revealed increased levels in the 2nd biopsy obtained from miscarriage, but not infertile patients.

(ii) In-depth characterization of primary stromal cell cultures before IVF treatment showed that disordered temporal changes in the secretome of decidualizing cultures are associated with subsequent implantation failure.

(iii) Characterization of perivascular eMSCs, which drive endometrial regeneration, highlighted the unique properties of these cells in terms of gene expression, metabolism, and clonogenic and angiogenic potential.

(iv) eMSCs also formed 3D structures that resemble the uterine mucosa when cultured in Matrigel. These novel organoids termed endometrial regenerative bodies (ERBs), epithelialize when co-cultured and decidualize in response to differentiation cues.

6.1 Future directions

One of the most striking findings of my thesis is the significant increase in endometrial clonal cell population following luteal phase biopsy in a subset of women with a history of miscarriage. This supports recent findings that endometrial stem cell deficiency and heightened cellular senescence are associated with recurrent pregnancy loss (Lucas et al., 2015). Furthermore, a randomised controlled pilot study is now underway at University Hospitals Coventry and Warwickshire. In this study, 100 miscarriage patients without a history of conception delay are randomised to either mid-luteal endometrial biopsy or mock biopsy. The primary study outcome is miscarriage rate of pregnancies conceived within three cycles. It is anticipated that the study will be completed in late 2017.

Decidualizing endometrial stromal cells (EnSCs) surround the implanting embryo, but their role in implantation failure is poorly understood. Our results show that the temporal changes in the secretome of decidualizing EnSCs underpin the transition of a hostile to a supportive endometrial microenvironment. In addition to that, the perturbation in this transitional decidualization pathway precedes implantation failure. Thus, the endometrial defects associated with implantation failure are present before ART; it has potential clinical ramifications. It raises the prospect that screening could minimise the risk of subsequent treatment failure.

Amongst the highly enriched perivascular genes, a potential marker could be identified for eMSCs. Furthermore, ERBs could represent a unique new resource to study an array of endometrial disorders in a patient-specific manner. It could be developed into a model to define mechanisms involved in embryo implantation failure and to uncover molecular mechanisms that predict and prevent persistent reproductive failure. However, multiple further characterizations are required before this becomes a potential therapeutic option.

Although my work has begun to answer some important questions, it has also raised many new questions. If the trauma influences the clonal population, what effect does it have on other populations with the endometrium? As an expansion of the clonal population following trauma was not observed in infertile patients, does implantation failure reflect an imbalance in the various cellular ratios? As I have shown that the secretome is a temporal signature of implantation, it would be interesting to determine the transcriptomic map of the patient groups from this study, which could potentially lead to the expression of an aberrant secretome. Finally, further work with ERB formation could potentially reveal differences in patient groups which in turn may depend on the quality of the stem cells and niche within these patients.

In summary, the evidence presented in this thesis demonstrated that dyshomeostasis between stromal subpopulations, which may be caused by eMSCs deficiency or dysfunction, precedes reproductive failure.

APPENDICES

Appendix 1. List of primers

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
SELE	ACC AGC CCA GGT TGA ATG	GGT TGG ACA AGG CTG TGC
POSTN	CAC CAA GGT CAC CAAATT CAT	TTC CTC ACG GGT GTG TCT C
ANGPT2	TGC AAA TGT TCA CAA ATG CTA A	AAG TTG GAA GGA CCA CAT GC
DCLK1	CTC AGG CAA GTC GCC AAG	AAG AGA GGG GGC GGT ACA
F2RL3	GCT GCT GCA TTAC TCG GAC	ACG TAG GCA CCA TAG AGG TTG
EPHA3	CTGGATTCAAAAACAATTCAAGG	TCCACACCACTGATCTCTTCC
PDE1A	GCATACAGGGACAACAAACAAC	TCTCAAGGACAGAGCGATCAT
GUCY1A2	GAGGACTGCTTCGAGATTGTATC	TGGTTCTAATCACAAACGGGGTA
OLFML2A	CACGCCTACGTCCACAAGG	TCATAGTGCCTCAACTGCTCA
CCDC102B	TCATGGGCTACAATCTCATGCT	CGCAGGCGAAGTTCTTCAC
CNTN1	TGTTGAGGCTTCCGACAAAGG	AATGAGTGGGATGAATTTGCTGA
EDIL3	AGCATACCGAGGGGATACATT	CAAGGCTCAACTTCGCATTCA
L19	GCGGAAGGGTACAGCCAAT	GCAGCCGGCGCAAA
SUSD2	CTGGATGGACCTGAAAGGAA	AGCATGATGGAGACCCTGTC
PRL	AAG CTG TAG AGA TTG AGG AGC AAA C	TCA GGA TGA ACC TGG CTG ACT A
IGFBP1	CGA AGG CTC TCC ATG TCA CCA	GT CTC CTG CCT TGG CTA AAC
ESR1	CCCACTCAACAGCGTGTCTC	CGTCGATTATCTGAATTTGGCCT
PGR	ACCCGCCCTATCTCAACTACC	AGGACACCATAATGACAGCCT
11HSDB1	AGC AAG TTT GCT TTG GAT GG	AGA GCT CCC CCT TTG ATG AT

Appendix 2. Downregulated genes in ePCs (P < 0.05)

Gene symbol		Fold change (log 2)	*P*-value
WDFY4		4.09	0.0001
SERPINB2		5.99	0.0005
GBP5		2.55	0.0005
CD3D		4.76	0.0006
CYTIP		5.59	0.0006
CD84		4.44	0.0012
SCML4		6.85	0.0013
CD96		5.42	0.0013
IL1B		4.97	0.0014
ITGB7		2.62	0.0022
LCK		3.71	0.0025
CXCL9		2.16	0.0029
HMHA1		3.08	0.0029
CTLA4		4.19	0.0030
MSR1		5.47	0.0032
ICOS		6.37	0.0033
EMB		4.80	0.0038
ZNF831		5.10	0.0041
EREG		3.81	0.0041
PLEK		4.78	0.0050
CCL4		5.56	0.0053
IKZF3		3.18	0.0056
PIK3R5		6.20	0.0057
TLR2		2.08	0.0058
LAT2		3.61	0.0058
ITK		7.88	0.0061
CD52		3.88	0.0061
PTPRC		6.24	0.0061
IFI30		2.80	0.0062
VAV3		2.26	0.0063
CST7		3.98	0.0063
CD53		2.54	0.0064
FGD3		4.41	0.0067
OLR1		5.24	0.0068
C20orf26		3.08	0.0071
SELL		4.58	0.0073
ARRB2		2.56	0.0073

SLFN12L		3.78	0.0074
KLRC2		7.44	0.0075
ITGAM		3.97	0.0076
CLEC7A		6.58	0.0077
CCR5		4.25	0.0078
NFAM1		5.29	0.0079
LCP1		2.47	0.0081
TARP		6.32	0.0082
CD300E		4.93	0.0084
P2RY10		5.73	0.0086
CYFIP2		2.84	0.0087
KIF21B		2.77	0.0087
DOCK2		7.18	0.0088
LAIR1		2.90	0.0090
SLA2		2.65	0.0090
BIN2		4.75	0.0094
CCL3		5.81	0.0094
CTSS		2.06	0.0095
IL2RG		5.92	0.0097
MS4A7		6.10	0.0101
FASLG		5.26	0.0102
HLA-DQA1		4.92	0.0103
LY86		4.35	0.0103
GATA3		4.39	0.0104
CXCR4		2.36	0.0105
FMNL1		2.47	0.0109
HCAR3		4.52	0.0113
MYO1G		5.57	0.0113
FOLR2		7.51	0.0115
CD3E		7.38	0.0115
MAP4K1		3.69	0.0118
HLA-DRA		2.84	0.0119
CCR1		4.44	0.0120
FCGR2A		5.95	0.0123
PIK3CG		2.57	0.0128
THEMIS		4.40	0.0132
GPR174		5.95	0.0132
CD8A		4.77	0.0132
C1orf162		2.64	0.0135
GPR183		4.45	0.0136
EOMES		7.74	0.0141
GPRIN3		1.92	0.0141

CD300A		3.09	0.0144
PIK3AP1		5.29	0.0147
CD163		8.33	0.0148
SLA		5.01	0.0148
DOCK8		2.97	0.0156
SP140		3.89	0.0156
ITGAL		5.65	0.0157
HAVCR2		5.14	0.0157
HCLS1		2.67	0.0157
RASSF5		3.54	0.0158
IGJ		5.79	0.0162
KLRD1		4.23	0.0164
SELPLG		3.45	0.0174
AOAH		3.80	0.0176
CD244		4.42	0.0177
LAPTM5		3.85	0.0178
CD209		4.43	0.0179
PYHIN1		5.37	0.0179
NCKAP1L		6.74	0.0183
SAMSN1		5.59	0.0186
THEMIS2		2.82	0.0187
IL21R		4.95	0.0191
GPR65		5.58	0.0191
MS4A4A		6.10	0.0192
PRKCB		5.65	0.0193
SCN9A		3.95	0.0200
PTK2B		2.24	0.0200
CRTAM		5.14	0.0200
SLAMF7		4.95	0.0202
SH2D1B		3.12	0.0202
NCF4		4.94	0.0204
LCP2		1.66	0.0215
ARHGAP15		3.45	0.0219
DTHD1		3.07	0.0225
PARVG		3.97	0.0225
MPEG1		4.03	0.0228
GNG2		2.54	0.0229
HCAR2		3.56	0.0231
TAGAP		4.35	0.0232
ALOX5AP		4.74	0.0232
CD200R1		5.68	0.0235
PTGER4		3.49	0.0237

TNFSF8		3.10	0.0241
CD2		5.78	0.0243
WAS		3.88	0.0243
SCN3A		3.41	0.0245
VSIG4		5.94	0.0245
CD86		6.96	0.0247
IFNG		5.41	0.0247
IRF8		1.79	0.0247
IL10RA		4.87	0.0250
CLEC5A		3.57	0.0252
IL18RAP		5.32	0.0253
SLFN13		2.29	0.0254
PLCG2		1.87	0.0259
MYB		2.74	0.0261
IKZF1		5.41	0.0267
CD1E		5.46	0.0269
TYROBP		6.62	0.0271
GFI1		3.51	0.0274
KLRC3		4.65	0.0274
KLRB1		4.99	0.0275
BCL2A1		2.11	0.0281
SRGAP3		2.15	0.0284
PLCB2		3.91	0.0287
CD14		3.87	0.0288
CARD11		4.43	0.0290
CD69		3.28	0.0291
MS4A1		5.84	0.0293
CYBB		5.99	0.0302
AIF1		5.20	0.0303
NCAM1		3.02	0.0304
PTPN6		2.21	0.0306
ATP8B4		2.84	0.0307
CAPG		2.71	0.0309
MS4A6A		7.07	0.0313
GZMK		5.48	0.0322
RUNX3		6.04	0.0324
ARHGAP9		3.97	0.0325
RGS1		6.37	0.0326
HCK		4.26	0.0335
KLHL6		3.12	0.0343
B3GNT7		2.66	0.0343
PTPN22		4.29	0.0351

NKG7		6.09	0.0360
MYBL1		2.76	0.0362
GZMB		7.65	0.0363
FCGR3A		5.66	0.0365
RAC2		6.18	0.0366
APOBR		4.73	0.0367
WDR65		4.35	0.0374
TESPA1		5.88	0.0376
MRC1		4.01	0.0380
CLEC12A		5.13	0.0389
STAT4		3.46	0.0392
CXCR3		4.92	0.0398
SORL1		1.81	0.0400
C3AR1		3.76	0.0402
XCL1		7.59	0.0408
BCL11B		5.68	0.0410
CDHR1		2.84	0.0412
CYTH4		4.93	0.0415
HPGDS		5.24	0.0433
LY9		4.40	0.0434

Appendix 3. Upregulated genes in EnSCs (P < 0.05)

Gene symbol		*P*-value
RHOH		0.029682
APOBEC3H		0.034655
MATK		0.0297
LST1		0.022994
SEMA3D		0.003382
GZMH		0.033204
SIGLEC10		0.025167
CD48		0.01013
SIRPG		0.013398
KLRC4		0.017714
CD6		0.023111
SH2D1A		0.043566
CD300LB		0.041055
MS4A2		0.04327
VAV1		0.001838
TBX21		0.001865
ZNF683		0.028305
LILRB4		0.014437
ADAMDEC1		0.042495
CD3G		0.038125
SLAMF1		0.009059

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